ISOENZYMIC STUDIES OF ESTERASE, L-ASPARTATE: 2-OXOGLUTARATE AMINOTRANSFERASE, AND CARBOHYDRASE, IN *ZEA MAYS*

A DISSERTATION SUBMITTED TO THE
GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN HORTICULTURE

JANUARY 1969

By

Timothy Macdonald

Dissertation Committee:

James L. Brewbaker, Chairman
Geoffrey C. Ashton
John W. Hylin
Haruyuki Kamemoto
Yoneo Sagawa

We certify that we have read this dissertation and that in our opinion it is satisfactory in scope and quality as a dissertation for the degree of Doctor of Philosophy in Horticulture.

DISSERTATION COMMITTEE

James L. Brewbaker
Chairman

H Kamemoto

Yoneo Sagawa

John W Hylin

Geoffrey L Ashton

ACKNOWLEDGMENT

During the first three years of my doctoral program I was the recipient of a Title IV National Defense and Education Act Fellowship. I wish to thank the sponsoring agency involved for the financial assistance. I wish to personally thank Dr. Geoffrey Ashton for arranging continued support for my program in the form of an Assistant in Research position in the Department of Genetics at the University of Hawaii. Special thanks are due Dr. James L. Brewbaker. Had it not been for Federal Grants held by Dr. Brewbaker, the reagents and equipment required in this study would not have been available.

I also wish to thank Dr. Robert E. Parks, Jr. and Dr. Ralph P. Miech of the Division of Biological and Medical Sciences at Brown University for a most rewarding summer of research spent there during the development of my thesis program.

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF TABLES (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

INTRODUCTION

Isoenzymes have been the object of intense studies in both plants and animals for the past ten years. Two recent and comprehensive reviews have been written on animal isoenzymes (Latner, 1967) and plant isoenzymes (Shannon, 1968), and isoenzymes have been the object of a recent book (Wilkinson, 1966). The major technique used in the study of isoenzymes has been gel electrophoresis; a technique that was first described by Smithies (1955) and which has undergone comparatively little change since its first descriptions.

The term isozyme was first introduced by Markert and Møller (1959) to describe the different molecular forms of proteins which exhibit the same enzymatic specificity. In 1964, the term isoenzyme was adopted by the Standing Committee on Enzymes of the International Union of Biochemistry (Webb, 1964) as representing the multiple forms of an enzyme. The present concept of the term isoenzyme is as yet still rather broad in that precise criteria which must be fulfilled for two enzymes to be considered isoenzymes have not been established. At present then, the term isoenzyme refers to the existence of two or more forms of an enzyme within a single species.

Gel electrophoresis has become an important tool in defining the action of the gene. Prior to the development of this technique,

mutations resulting in differential gene function and the subsequent alterations in enzyme production were limited to impairment of enzymatic activities. Mutations, therefore, that altered but did not inactivate the enzyme went undetected. Schwartz (1960, 1962a, b, 1964a,b,c,d,e, 1965, 1967) has described seven alleles for the pH 7.5 esterases in maize. The different alleles specify esterase isoenzymes having different migration rates in starch gels. If an analysis on the basis of esterase activity had been the approach used in the study of the maize pH 7.5 esterases these different allelic forms would not have been detected. Other examples similar to this have been described (Latner, 1967; Shannon, 1968).

Regulation of the primary products of the gene (enzymes) has largely been confined to work with microorganisms (Jacob and Monod, 1961; Cline and Bock, 1966; Vogel and Vogel, 1967). Repression and induction models which involve regulator and operator genes have come from such studies. The concept of the operon containing a discreet operator cistron has been postulated. In higher organisms, on the other hand, there is an obvious lack of data with respect to the operon theory. Schwartz (1962) has postulated the existence of 'prime' alleles and 'standard' alleles for the pH 7.5 esterases in maize. The 'prime' alleles of this system are subject to regulation in the form of cessation of enzyme production at certain stages of development, while the 'standard'

alleles are not subject to this regulation. The system of 'prime' and 'standard' alleles is analogous in some respects to the operon theory. Criteria for specifying a discreet operator cistron in this system have not been met largely because linkage between the regulatory portion of the 'prime' allele and the functional or structural portion of this locus has not been broken.

The present study grew out of a classification of esterase polymorphism in the species Zea mays. Three hundred to four hundred inbreds, varieties, and races of maize were examined for esterase activity. A general description of the anodal esterase isoenzymes occurring in maize tissues is presented. The maize esterase isoenzymes were also studied on the basis of substrate specificity, inhibition and activation, changes occurring in the isoenzymic pattern during development, and genetic analysis. A similar, but limited, study was made for maize transaminase isoenzymes and several carbohydrase isoenzymes.

It will be shown that the maize esterases constitute a heterogeneous and complex mixture of isoenzymes which show a certain amount of tissue specificity, variations in substrate utilization, variations in responses towards inhibitors and activators, and changes in the spectrum of isoenzymes during germination. In addition, nine new genetic loci are described for the esterase isoenzymes. Simple and complex genetic mechanisms are noted, and in one instance regulation of enzyme production resulting in a

model somewhat analogous to a 'prime' and 'standard' allele was found. A hybrid enzyme was found to be produced in heterozygotes involving two variant transaminase isoenzymes.

MATERIALS

Materials utilized in the present study were obtained from a world collection of approximately 500 inbreds, varieties, and races of Zea mays maintained at the University of Hawaii, Honolulu, Hawaii. In cases where specific crosses were required, the material was grown either on the Manoa campus or at the experimental stations located at Waimanalo or Poamoho.

Various tissues of the seed, seedling, and mature maize plant were used as enzyme sources throughout the study. In one study involving phosphorylase isoenzymes, potato tubers obtained from the local markets was used as a source of enzyme. Tissues from seedling up to 10 days of age were obtained from plants grown in the laboratory. Seed was germinated in the dark and transferred to the benchtop on the third day of germination. The seeds were germinated in petri dishes which contained moistened filter papers. Older tissues were collected from plants grown in the field.

Laboratory facilities at the Department of Horticulture of the University of Hawaii were used to conduct the research.

Chemical reagents were obtained through commercial supply houses.

GENERAL METHODS

Seed increase and specific crosses involving the maize material were made by hand pollinations. Tassels were covered the afternoon before the pollinations were made and pollen was collected on the day of pollination. Ears of the maize plant were covered prior to the appearance of silks and were pollinated when the silks had grown approximately 5 cm beyond the husks. The newly pollinated ears were covered and labelled to indicate the cross involved.

Horizontal gel electrophoresis was employed in the study of maize isoenzymes. The method originally described by Smithies (1955) was followed with slight modifications (Brewbaker, Upadhya, Makinen and Macdonald, 1968). Both starch and polyacrylamide gels were utilized.

Starch gel preparation: Starch gels contained 13.5% hydrolysed starch (Connaught Medical Research Laboratories, University of Toronto, Canada). Thirty ml 126-A (Table 1) were mixed with 270 ml 126-B (Table 1). One hundred ml of this mixture were added to a liter pyrex flask containing 40 g of starch. The remaining 200 ml of buffer solution were brought to a boil and mixed well with the starch solution to produce a viscous solution (final pH 8.2). The flask was evacuated using a faucet aspirator until removal of gases was essentially complete and the

Table 1. Buffer Solutions Used in the Present Study

Buffer	pH	Components
Tris(Cl)	7.5	0.1 M Trizma Base* adjusted to pH 7.5 with concentrated HCl.
Tris-citrate	6.2	0.1 M Trizma Base adjusted to pH 6.2 with 0.1 M citric acid.
Phos-A	8.8	0.2 M dibasic sodium phosphate.
Phos-B	4.6	0.2 M monobasic sodium phosphate.
126-A	8.1	0.025 M lithium hydroxide. 0.2 M boric acid.
126-B	8.2	0.01 M citric acid. 0.065 M Trizma Base.

*tris(hydroxymethyl)aminomethane-HCl

material boiled smoothly. The gel material was then poured into plexiglass trays having the dimensions 18 cm x 20 cm x 3.2 mm and containing a 3.2 mm border. The trays accommodated a starch block approximately 6.5 mm in thickness and allowed the gel to be cut into identical halves following electrophoresis by drawing a cheese cutter through the gel parallel with the bottom of the tray. Starch gels hardened sufficiently for manipulation within 40 minutes of pouring.

Acrylamide gel preparation: The acrylamide gels were prepared using the same buffers and the same trays used in the preparation of starch gels. Seven g. of Cyanogum-41 (Fisher Scientific Co.) were added to 10 ml 126-A and 90 ml 126-B and dissolved. One ml of a 10% aqueous solution of ammonium persulfate and 0.2 ml of N,N,N',N'-tetramethylethylene-diamine (Eastman Organic Chemicals) were added to the buffer solution and the mixture was poured into the tray. The tray was then covered with saran or a glass plate being careful not to trap air bubbles between the covering and the solution of gel material. The gel hardened within 20 minutes.

A continuous origin slot was cut in the starch gel by drawing a knife vertically through the gel approximately 5.5 cm from the cathodal end of the gel. Individual origin, or sample, slots were cut into the acrylamide gel using a razor blade that had been cut to the size of the sample wick to be inserted. A vertical

pressure was applied to the razor blade pressing it through the gel to the bottom of the tray. It was found that a slicing motion tended to tear the acrylamide gel. The samples were applied to the gel by first absorbing extract material into paper wicks cut to the desired size and then placing the wick directly into the origin slot. Wick size depended upon the number of samples applied to the gel. The samples were found to give best resolution if the wicks did not extend above the surface of the gel A distance of approximately 1.5 mm was allowed between sample wicks in the starch gel and approximately 3 mm between sample wicks in the acrylamide gel.

The electrode tanks contained 126-A (Table 1). Sufficient buffer (electrolyte) was added to the tanks to allow soaking of the sponge bridges and to well cover the electrodes. Tank electrolyte buffers were mixed following each electrophoretic run and were replaced with fresh buffer solution every two electrophoretic runs.

The gel was subjected to electrophoresis under an applied voltage gradient of 6-8 V/cm for a period of five hours, or until the brown borate front (visible in starch gels for most tissues) had migrated 8.5 cm towards the anode from the origin slot. In the case of acrylamide gels, the front was visualized by inserting a sample wick saturated with Sheaffer's blue ink (#42). A visible portion traveled along with the borate front towards the anode.

The gels were placed on the electrode tanks and the sponge bridges were pulled up and rested on the ends or the gel. The gel and sponges were covered with saran to avoid water loss and a glass plate was then placed on the covering saran to hold the sponges in place. The whole apparatus was contained in a refrigerator and electrophoresis was carried out at 7°C. Power was supplied from a Heathkit Model IP-32 power supply located outside of the refrigerator. Following electrophoresis, the gels were removed from the apparatus and cut into the appropriate sizes for staining or treatment.

Preparation of the samples: Fresh tissue samples were ground with an equal volume of extraction solution. Grinding continued until the sample was smooth and homogeneous in appearance. A small piece of tissue was often applied to the extracted tissue to act as a filter between the tissue and the sample wick, however this did not appear to improve resolution so was not always used. Sample wicks consisted of chromatographic grade filter paper cut to the appropriate dimensions. Varying thicknesses of filter paper were tried and all were found to be suitable as sample wick material. Extraction was carried out at room temperature since it was found that extraction in the cold room did not improve resolution.

Several extraction solutions were employed. It was generally found that a saline solution (0.8% NaCl, 0.2% $NaNO_3$)

produced suitable resolution. Tris(Cl) (Table 1) was also used with equal success. In certain instances which will be mentioned below, cysteine (10^{-3} M) was included in the Tris(Cl) extraction solution.

When immature endosperm was used as the tissue source for enzymes, the kernel was punctured with a needle and the milky endosperm was absorbed into the sample wick directly.

Staining techniques and any modifications of the methods described in this section will be described under the different enzyme sections.

ESTERASE ISOENZYMES

1. Introduction and Staining Technique

The esterases are a complex group of hydrolases generally exhibiting a rather low substrate specificity. Three major groups of esterases exist: (1) fat splitting enzymes, which act on glycerol esters (true lipases), (2) the lipase-type esterases, which act on predominantly undissolved substrates, and (3) the ester hydrolases proper, or the esterases which act on dissolved substrates. Generally speaking, the reaction involves the hydrolysis of an ester resulting in the production of a monocarboxylic acid and an alcohol. The lipases presumably split triglycerides to produce fatty acids and glycerol, hence their role involves lipid metabolism. The many other esters occurring in biological systems are presumably acted upon by the other esterases. Cholinesterases have generally been associated with nerve impulse transmission due to their affinity toward acetylcholine. The physiological role of the esterase isoenzymes found in the present study is unknown. Certain of these esterases appear to be capable of hydrolysing certain lipid-like substrates which may indicate a role in lipid metabolism, especially in tissues rich in lipids such as the scutellum. The present study involved a cataloging of the esterase isoenzymes found in the maize plant. Substrate specificity and inhibition and activation studies were conducted in order to better

define the maize esterase isoenzymes. The genetic control of the esterase isoenzymes was also carried out.

Electrophoresed gel slices were immersed in a solution consisting of 100 ml 0.1 M phosphate buffer (pH 6.5) which contained 100 mg Fast Blue RR Salt (Sigma Chemical Co.) and 3 ml of a 1% α-naphthyl acetate solution. The phosphate buffer was prepared by mixing one part Phos-A, five parts Phos-B (Table 1), and six parts distilled water. The 1% α-naphthyl acetate solution was prepared by adding 1 g of the substrate to 100 ml 70% ethanol. The substrate solution could be stored for several months under refrigeration without noticeable changes in the staining reaction.

One hundred mg of Fast Blue RR Salt were added to 100 ml phosphate buffer and mixed. The mixture was filtered to remove residual undissolved salt. Following filtration and just prior to use of the stain, 3 ml of the substrate solution was added and mixed. The gel slice was immersed in this staining solution with the cut surface of the slice facing away from the bottom of the staining tray. The tray was then placed in an incubator at 37°C for approximately one hour, or until the esterase isoenzymes had stained dark enough to allow classification. The esterase isoenzymes stained in this manner appeared blue-black in color.

Following the staining process, the stain was poured off and the gel slice was rinsed with water. The gel slice was then

transferred to a fixing solution composed of methanol:acetic acid: water (6:1:4). Four hours or more in the fixing solution generally hardened the gel and turned the starch white. This allowed for ease in handling and a greater contrast between the blue-black isoenzymes and the white starch background.

2. Description and Occurrence

Approximately 32 isoenzymes from maize tissues capable of hydrolysing α-naphthyl acetate were found on the anodal side of pH 8.2 starch gels. Mobility values (Rm) were assigned to the different isoenzymes relative to an intensely staining isoenzyme which was found to be present in all tissues studied. The reference isoenzyme (isoenzyme 18) (Rm 100) occurred at an average Rf value (relative to the brown borate front) of 84 with a coefficient of variation of 1.16% in 100 gels.

Tissues examined in the present study were root, coleoptile, plumule, endosperm, and scutellum of the developing seedling, mature leaf, root, pollen, nodes, internodal pith, husk, mature and immature ears, tassel branches, anthers, and immature endosperm from developing kernels.

Figure 1 illustrates the anodal esterase isoenzymes found to be present in the different tissues of maize. The figure represents a composite of all of the esterase isoenzymes encountered in the 300 inbred, varieties, and races of maize examined. A single

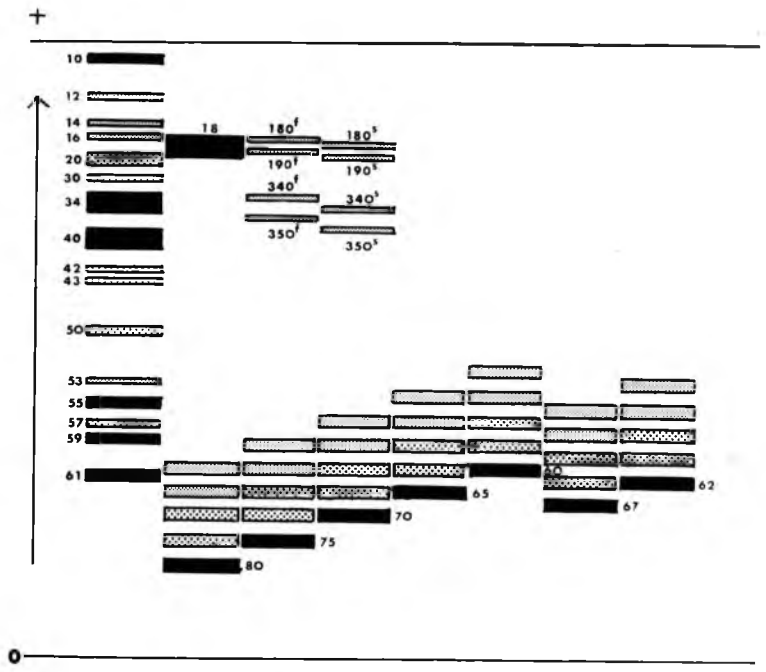

Fig. 1. An Illustration of the Anodal Esterase Isoenzymes of Maize. The illustration represents a composite of esterase isoenzymes found in 17 tissues and 300 lines of Zea mays. The origin is at the bottom and the line at the top represents the approximate position of the borate front. The arrow indicates migration towards the anode.

inbred of maize would contain only certain of these isoenzymes. Table 2 illustrates the tissues in which the isoenzymes occurred.

Isoenzyme 10 had a Rm value of 117 and appeared only in seedling roots that had been infected by fungus (Fusarium sp.). It was first observed in root tissue from seedlings which were 12 to 15 days old and was thought to be an age dependent isoenzyme. Seedlings were grown in the presence and absence of the fungicide Panogen and examined at different stages of growth. It was found that only seedling root tissue from fungicide free seedlings contained this isoenzyme. Mycelia from the fungus did not contain isoenzyme 10 and it was not determined whether this isoenzyme was produced by the fungus due to its association with roots or by the roots due to infection.

Isoenzyme 12, (Rm 110), was found in most tissues examined. It was a relatively weak isoenzyme and difficult to classify in some tissues.

Isoenzymes 14 and 16, (Rm 105 and 102), occurred in several of the tissues examined and in most of the lines of maize tested. They always occurred together and were never seen singly in any tissue.

Isoenzyme 18, (Rm 100), was a strongly staining isoenzyme occurring in all tissues examined. The majority of the lines studied contained this isoenzyme, however, it was noticeably missing in some lines. Slight variations in mobility of this

Table 2. Occurrence of Esterase Isoenzymes in Different Tissues of Maize

Isoenzyme	Tissues* 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
10	s**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	w	w	w	0	0	m	w	w	0	0	0	w	0	w	0	0	0
14-16	w	w	0	m	w	0	0	0	0	0	0	w	0	0	w	w	0
18	s	s	s	v	s	s	s	s	s	s	s	s	s	s	s	s	s
Complex-I, II	m	m	0	m	0	0	0	0	0	0	0	0	0	0	0	w	0
20	m	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
30	m	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	s	s	s	v	w	s	s	s	s	s	s	s	s	s	s	s	s
40	s	s	s	v	w	s	s	s	s	s	s	s	s	s	s	s	s
42-43	m	w	w	v	w	w	w	w	w	w	w	w	w	w	w	w	w
50	0	0	0	0	0	m	0	0	0	0	0	0	0	0	0	0	0
53	0	0	0	0	0	w	0	0	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	s	0
57	0	0	0	0	0	m	0	0	0	0	0	0	0	0	0	0	0
59	0	w	0	w	0	0	0	0	0	0	0	0	0	0	0	s	0
61	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	s	0
60-80	s	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

*1 = seedling root
2 = plumule
3 = coleoptile
4 = scutellum
5 = mature endosperm
6 = immature endosperm
7 = mature leaf
8 = mature root
9 = node
10 = pith
11 = husk
12 = immature ear
13 = mature ear
14 = tassel branch
15 = mature anther
16 = pollen
17 = adventitious root

**w = weak, m = medium, s = strong, v = very strong staining isoenzymes, 0 = no stain, - = not tested.

isoenzyme were noted between several inbred lines. The variation in mobility was insufficient, however, to accommodate genetic analysis.

Isoenzymes 180^f, 180^s, 190^f, 190^s, 340^f, 340^s, 350^f, and 350^s, (Rm 101, 100, 99, 98, 90, 88, 86, and 84, respectively), were medium to weak staining isoenzymes found only in root, plumule, and scutellum tissues of the young seedling and in pollen tissue. An inbred line of maize always contained four of these isoenzymes. All but one inbred line examined in the present study contained isoenzymes 180^f, 190^f, 340^f, and 350^f. This group if four isoenzymes was assigned the designation Complex-I. The single inbred line not containing Complex-I contained isoenzymes 180^s, 190^s, 340^s, and 350^s. This group of four isoenzymes was designated as Complex-II. As will be shown below in the section on genetic control isoenzymes of Complex-I were never found to occur with isoenzymes of Complex-II.

Isoenzyme 20, (Rm 98), was found in 3-day old seedling root tissue of inbred 382 (Purdue) only. At later stages of development this isoenzyme disappeared.

Isoenzyme 30, (Rm 94), was a weakly staining isoenzyme found in an introduction line from Bolivia (PI 240320). It was not seen in any other lines examined. Isoenzyme 30 was never isolated in a homozygous background and was not studied genetically.

Isoenzyme 34, (Rm 89), was an intense staining isoenzyme found in all tissues examined. It occurred in approximately 50% of the lines tested and always occurred independently of isoenzyme 40 in inbreds.

Isoenzyme 40, (Rm 82), like isoenzyme 34, was a rather intense staining isoenzyme and occurred in approximately 50% of the lines tested. It occurred in all tissues examined.

Isoenzymes 42 and 43, (Rm 76 and 74), were found in all tissues examined and all lines tested. Slight variations in the strength of these isoenzymes occurred in certain inbreds, but convincing null types were not encountered.

Isoenzymes 50 and 57, (Rm 64 and 46), occurred only in immature endosperm from developing kernels. They always occurred independently of each other and inbreds lacking both isoenzymes were not found.

Isoenzyme 53, (Rm 54), was a weak isoenzyme found only in immature endosperm of developing kernels. It was present in all lines tested.

Isoenzymes 55, 59, and 61, (Rm 50, 45, and 36), were strong isoenzymes found in pollen. A single weak isoenzyme, corresponding in mobility to isoenzyme 59, was noted in scutellum and plumule tissue of seedlings. It was not determined if this weak staining isoenzyme found in the seedling tissues corresponded to isoenzyme 59. Otherwise, these three isoenzymes were

specific to pollen tissue.

Isoenzymes 60, 62, 65, 67, 70, 75, and 80, (Rm 37, 35, 33, 31, 29, 24, and 19), were restricted to seedling root tissue. These isoenzymes demonstrated a unique multiplicity which varied from a single isoenzyme to eight isoenzymes within a single inbred line. Several sweet corn inbred lines 2253 (Iowa), C42 (Minn.), S4 (Minn.), S8 (Minn.), 338 (Purdue), T35 (Ill.), and 318a (Ill.) did not contain any of these isoenzymes. An inbred line contained only one of these groups of isoenzymes at a time. That is, if the group designated as isoenzyme 60 occurred in a particular inbred, all other groups were missing.

The phenotypes contained a major dark staining isoenzyme (the slowest migrating isoenzyme of the group) and up to seven faster and weaker isoenzymes. As can be seen from Figure 1, the slowest migrating isoenzyme of group 75 corresponded with the second isoenzyme of group 80; the slowest migrating isoenzyme of group 70 corresponded with the second isoenzyme of group 75, etc. Groups 62 and 67 varied from this series of coincidences in that the major, or slowest, isoenzyme of group 62 fell between the major isoenzyme of group 60 and the major isoenzyme of group 65. The major isoenzyme of group 62 corresponded with the second isoenzyme of group 67.

Variations in the 'multiplicity' of these groups appeared to be a function of technique. The multiplicity could be reduced to a

single isoenzyme by extracting the root tissue in buffer containing 10^{-3} M cysteine. Examples of this type of multiplicity have been noted with malate dehydrogenase from chicken mitochondria (Kitto et al., 1966). Single isoenzymes isolated from a multiple series were treated with iodine. They showed that the isoenzyme could be induced to demonstrate a multiplicity through iodination. They postulated that conformational changes had occurred through the treatment of the single molecular form which resulted in the production of altered molecular forms. They called the different molecular conformations 'conformers'.

Harris (1966) found similar groups of esterase isoenzymes in seedling root tissue from corn and described them as the E_4 esterases. He went on to localize the E_4 locus on chromosome 3 (Harris, 1968). As in the present study, his groups were restricted to the root tissue. The mobility of his groups were in the same general region as those in the present study. It is suspected that the E_4 esterases studied by Harris (1966) correspond to groups 60 through 80 of the present study. By varying the concentrations of his gels and varying the pH at which electrophoresis was carried out, Harris (1966) concluded that the multiple factors (conformers) of each group had identical molecular weights. It would appear, therefore, that the multiplicity of these groups represent conformational changes in a single basic structure, and that inbreds containing different groups contain different

basic units. That is, the conformational changes that occur to produce the multiplicity within a certain group is a reversible change. The mechanism by which this change occurs operates on all groups. The variation between the major (basic) isoenzyme of each group is an expression of an irreversible conformational change.

Tissue specificity for the maize esterase isoenzymes was noted by Scandalios (1964). He reported the esterase isoenzymes from different tissues of inbred AA4 (Haw.). The isoenzymes that he reported correspond to several of the isoenzymes described in the present study. Several additional isoenzymes have been found in inbred AA4 in the present study.

Throughout the present study it was noted that scutellum tissue contained the greatest amount of esterase activity. Generally speaking, the isoenzymes from scutellum tissue were stronger than corresponding isoenzymes in other tissues.

Summarizing then, it was found that maize contained several tissue specific isoenzymes. The E_4 esterases, or isoenzymes 60 through 80 exclusive of isoenzyme 61, were found only in seedling root tissue. Isoenzymes 50, 53, and 57 were restricted to developing endosperm tissue. Isoenzymes 55 and 61 were found only in pollen. Several isoenzymes, 18, 34, 40, 42, and 43 were found in all tissues examined. Other isoenzymes were found in some tissues but not others.

3. Changes During Germination and Development

Mature seeds of selected maize inbreds representing the full complement of esterase isoenzymes were tested for their esterase activities at different stages of germination. Seeds were soaked for 24 hours in running tap water and transferred to petri dishes containing moistened filter paper. The petri dishes were maintained in the dark until the third day of germination at which time they were placed on the benchtop in the laboratory. The 24-hour soaking period represented the first day of germination.

Tissue samples from the first, third, fourth, fifth, sixth, and seventh days of germination were extracted and analyzed electrophoretically for esterase activity. Due to the small size of the root and plumule of seeds germinated for less than four days, only scutellum, endosperm, and whole embryo axis were analyzed at earlier stages of germination. All tissues were separated and washed with distilled water prior to extraction.

Figure 2 illustrates the spectrum of esterase isoenzymes from tissues of germinating seed of inbred AA6. Scutellum contained an isoenzyme (designated as x in the illustration) on the first day of germination that disappeared at later stages of germination. On the fifth day of germination a second isoenzyme appeared in scutellum (designated as y in the illustration) which was not present at earlier stages of germination. A large increase in total activity, indicated by strengthening of the individual

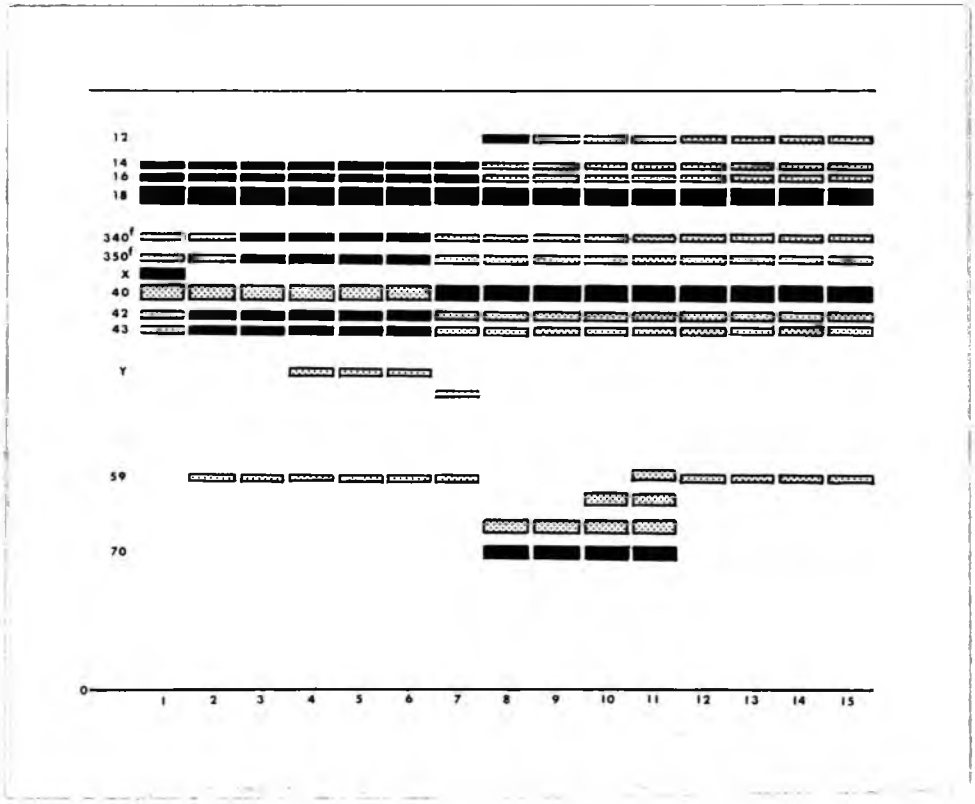

Fig. 2. Changes in the Esterase Isoenzyme Patterns of Tissues from Germinating Seed of Inbred AA6 (Haw.) During Development of the Seedling.

*Scutellum tissue of (1) 1-day old, (2) 3-day old, (3) 4-day old, (4) 5-day old, (5) 6-day old, (6) 7-day old seedlings; (7) embryo axis of 3-day old seedling; root of (8) 4-day old, (9) 5-day old, (10) 6-day old, (11) 7-day old seedlings; plumule of (12) 4-day old, (13) 5-day old, (14) 6-day old, and (15) 7-day old seedling.

isoenzymes in the scutellum, was seen as the seedling aged. The entire embryo axis of the 3-day old seedling showed a minor isoenzyme that was not present in either the root or plumule tissue at later stages of germination. Isoenzyme 18 was present in all tissues throughout the germination process. Isoenzymes 14-16 were also present in all tissues at all stages, however, a weakening of these two isoenzymes occurred in the root and plumule tissues at later stages of germination. Isoenzyme 12 was present in root and plumule tissues but diminished in strength as the seedling aged. Isoenzyme 12 was not seen in whole embryo axis on the third day of germination. Isoenzymes 42 and 43 became noticeably weaker in root and plumule tissues as the seedling aged. Isoenzyme 40 was present in all tissues throughout the germination process. The same was noted for isoenzyme 34 in a separate series.

Mature endosperm from dry seeds, seeds that had been germinated for one, two, three, four, and five days, was analyzed electrophoretically for esterase activity (Figure 3). The figure illustrates endosperm tissue from inbreds M119 and AA6. Isoenzyme 18 occurred throughout all stages of germination. Isoenzymes 14-16, present in inbred AA6, were not seen in the dry seed endosperm but appeared at later stages of germination. Isoenzyme 40 appeared throughout all stages of germination. Isoenzymes 42 and 43 were weak in dry seeds but became stronger as the seeds germinated. A large diffuse area of

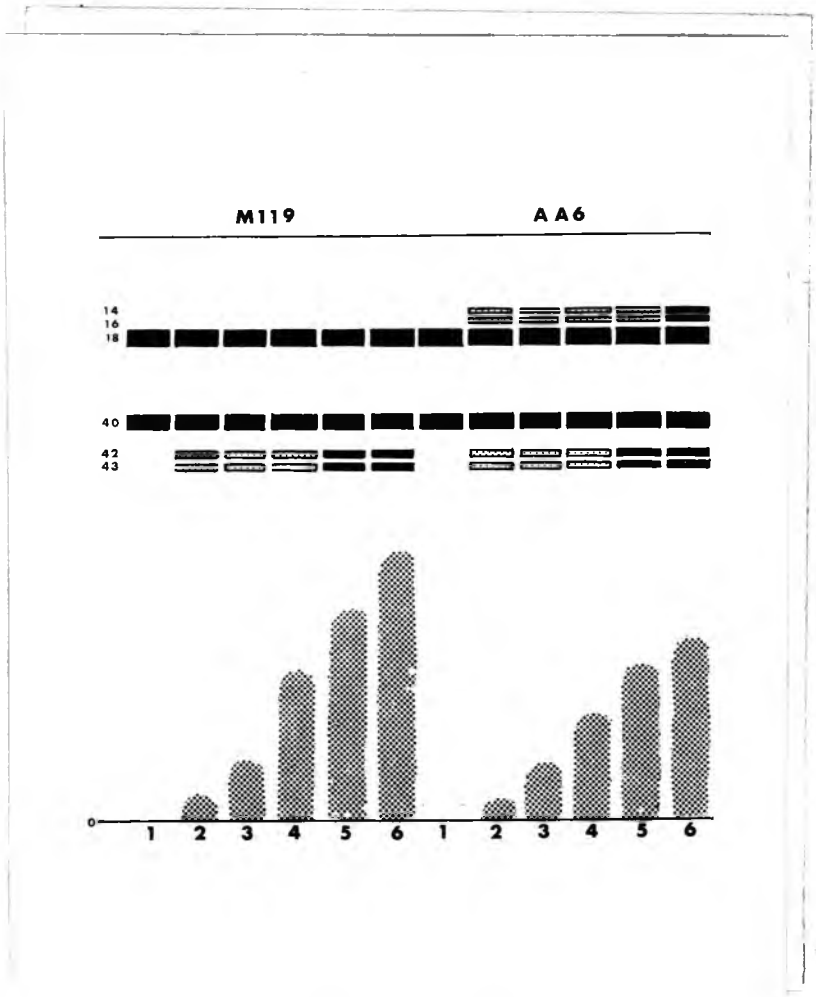

Fig. 3. Changes in Esterase Isoenzyme Patterns of Endosperm Tissue from Germinating Seeds of Inbreds M119 (Purdue) and AA6 (Haw.) During Development of the Seedling.

*Endosperm from (1) dry seed and from endosperm of (2) 1-day old, (3) 2-day old, (4) 3-day old, (5) 4-day old, and (6) 5-day old seedlings. Hatching extending from the origin at the bottom of the figure represents amylase activity.

esterase activity occurred in mature endosperm extending from the origin towards the anode throughout the germination process. As can be seen from the figure, large amounts of amylase activity occurred in later stages of germination.

One of the most dramatic features of germination is the disappearance of reserve materials. The great increase in esterase activity in the scutellum during the germination process may be correlated to these disappearances in some manner. During the germination process there is a conversion of fat to carbohydrate, the first step of which is the conversion of triglycerides to free fatty acids and glycerol. According to Beevers (1961a,b) this is accomplished by a greatly increased neutral lipase activity. The fatty acids thus produced are not accumulated but are converted to acetyl Co-A by β-oxidation (Stumpf and Barber, 1956). The acetyl Co-A is then converted to carbohydrates via the glyoxylate cycle and other metabolic pathways.

As much as 40% of the dry weight of the maize scutellum is composed of lipids (Zeller, 1957). Toole (1924) noted that before germination large globules of fat were present in the scutellum of maize. After germination, these fat globules had disappeared. At the same time, sugar and starch granules were noted to accumulate in the scutellum and embryo axis of the germinating seed. James and James (1940) and Albaum and Eichel (1943) showed that the loss of lipid material from embryos

(scutellum intact) of developing cereal grains preceded the mobilization of carbohydrate reserves in the endosperm. During the period of rapid fat breakdown, the RQ values were less than 1.0 suggesting that the lipids were serving as the respiratory substrate (James and James, 1940; Dure, 1960a). Data presented by Ingle et al. (1964) showed that scutellum tissue of germinating maize demonstrated a 173% increase in carbohydrate content and a 59% loss in lipid content over a period of five days. The greatest amount of lipid loss occurred in the last three days of the germination test (3rd, 4th, and 5th days of germination). This delayed utilization of lipid material agreed with the observation of Toole (1924) and Malhortia (1934) but contrasted with the observations of Dure (1960).

The glyoxylate cycle required for the conversion of the lipids to carbohydrates was found in scutellum of five-day old seedlings of maize (Oaks and Beevers, 1964b). Other maize tissues did not contain significant levels of glyoxylate cycle activities (Harley and Beevers, 1963).

The esterase isoenzymes of the maize scutellum may play an important role in the conversion of lipid materials to sugars. The increase in esterase isoenzyme activity of the scutellum of 5, 6, and 7-day old seedlings agrees with the observations of fat loss, during the same stages of development, presented by Ingle et al. (1964), Toole (1924), and Malhortia (1934).

It is tempting to assign lipase activity to these esterase isoenzymes due to the high degree of overlapping substrate specificity of esterases in general (see section on substrate utilization). A possible correlation was noted by Lewis and Hunter (1966) between α-naphthyl acetate hydrolyzing esterase isoenzymes and lipid metabolism in rats. Their experiments involved the examination of intestinal tissue in rats that had been fed on diets varying in lipid content. They showed that intestinal tissues from rats fed on corn oil diets contained significantly higher esterase activity than rats fed on corn oil free diets. The increased level of esterase activity in the lipid containing scutellum tissue of the present study may be an indication of their role in lipid metabolism.

4. Substrate Utilization

Extracts from selected inbreds representing 21 of the described isoenzymes were tested for their capacity to utilize substrates other than α-naphthyl acetate. Root and scutellum tissue from five-day old seedlings were used as esterase sources. Isoenzymes not tested in this series were 10, 20, 30, 50, 53, 57, 55, and 61.

The substrates tested in the present series were the alpha and beta forms of naphthyl acetate, naphthyl laurate, naphthyl myristate, naphthyl palmitate, naphthyl stearate, alpha naphthyl butyrate, and beta carbonaphthoxy choline iodide. The staining

solution described earlier for esterase staining was used in the present study with the substitutions of the different substrates. The concentrations of the substrates added to each test stain was the same (0.03%) as that described earlier for α-naphthyl acetate. One and one-tenth grams Fast Blue RR Salt was added to 1200 ml of 0.1 M phosphate buffer (pH 6.5) and filtered. The solution was divided into 12 100-ml portions and three ml of substrate solution was added to each portion. The final pH values of the solutions ranged from 6.2-6.5. The staining solutions were used in this form. Incubation times varied from 45 minutes to 15 hours depending upon the stainability of each solution. The longer chain esters (palmitate, stearate) showed limited solubilities in the test stains, hence the final concentrations of these substrates was somewhat lower than 0.03%.

The acetate and butyrate esters were hydrolyzed by all of the isoenzymes present in the extracts (Tables 3 and 4). The laurate and myristate esters were hydrolyzed to a limited degree by isoenzymes 18, 34, 40, 42, 43, and the 60-80 groups following 12 hours of incubation. The palmatate and stearate esters were hydrolyzed to a very slight degree in the scutellum tissues only after 15 hours. The isoenzymes of the scutellum that stained in these solutions were isoenzymes 18, 34, 40, 42, and 43. These isoenzymes stained extremely weak. The choline ester was hydrolyzed in both root and scutellum tissue following 4 hours

Table 3. Substrate Utilization by Esterase Isoenzymes Extracted from Scutellum Tissue

Substrate	Isoenzymes								
					Complex-				
	14-16	18	34	40	I	II	42	43	59
α-naphthyl acetate	m*	s	s	s	m	m	m	m	w
β-naphthyl acetate	m	s	s	s	m	m	m	m	w
β-naphthyl butyrate	w	s	m	m	w	w	w	w	w
α-naphthyl laurate**	0	m	m	m	0	0	w	w	0
β-naphthyl laurate**	0	m	m	m	0	0	w	w	0
α-naphthyl myristate**	0	m	m	m	0	0	w	w	0
β-naphthyl myristate**	0	m	m	m	0	0	w	w	0
α-naphthyl palmitate**	0	w	w	w	0	0	w	w	0
β-naphthyl palmitate**	0	w	w	w	0	0	w	w	0
α-naphthyl stearate**	0	w	w	w	0	0	w	w	0
β-naphthyl stearate**	0	w	w	w	0	0	w	w	0
β-carbonaphthoxy choline iodide	0	m	m	m	0	0	m	m	0

*w = weak, m = medium, s = strong, 0 = no staining.
**prolonged staining time.

Table 4. Substrate Utilization by Esterase Isoenzymes Extracted from Root Tissue

Substrate	Isoenzymes									
	12	14-16	18	34	40	Complex-I	Complex-II	42	43	60-80
α-naphthyl acetate	w*	m	s	s	s	m	m	w	w	s
β-naphthyl acetate	w	m	s	s	s	m	m	w	w	s
β-naphthyl butyrate	w	w	s	s	s	w	w	w	w	s
α-naphthyl laurate**	0	0	m	m	m	0	0	w	w	w
β-naphthyl laurate**	0	0	m	m	m	0	0	w	w	w
α-naphthyl myristate**	0	0	m	w	w	0	0	w	w	w
β-naphthyl myristate**	0	0	m	w	w	0	0	w	w	w
α-naphthyl palmitate**	0	0	0	0	0	0	0	0	0	0
β-naphthyl palmitate**	0	0	0	0	0	0	0	0	0	0
α-naphthyl stearate**	0	0	0	0	0	0	0	0	0	0
β-naphthyl stearate**	0	0	0	0	0	0	0	0	0	0
β-carbonaphthoxy choline iodide	0	0	m	m	m	0	0	w	w	w

*w = weak, m = medium, s = strong, 0 = no staining.
**prolonged staining time.

of staining by isoenzymes 18, 34, 40, 42, 43, and the 60-80 groups.

It was noted earlier that the scutellum contained the strongest staining isoenzymes. Isoenzymes 18, 34, 40, 42, and 43 were found to be especially strong in this tissue. This is perhaps the reason why only scutellum tissue was able to utilize the palmitate and stearate esters following prolonged incubation. There appeared to be a definite relationship between the length of the acyl group of the ester and the ability of the esterase isoenzymes to utilize the substrate. The longer chain esters (laurate, myristate, palmitate, and stearate) were hydrolyzed very slowly in comparison with the 2 and 4 carbon acetate and butyrate esters.

The zymogram has been used as a tool in substrate specificity studies for the esterases of mice (Hunter and Burstone, 1960; Eranko et al., 1962; Hunter and Strachan, 1961), rats (Schwark and Ecobichon, 1967), and humans (Ecobichon, 1966). It is generally agreed that the majority of the esterase isoenzymes do not hydrolyze the longer chain esters at significant rates. The chain lengths of the different naphthyl esters have, however, been used to differentiate between esterases and lipases (Nachlas and Seligman, 1949; Hunter and Strachan, 1961). Hunter and Strachan (1961) indicated that one of the isoenzymes they observed was a lipase on the basis that it hydrolyzed β-naphthyl laurate but not shorter chain substrates. Esterase isoenzymes

that hydrolyze both short chain esters and chains up to 18 carbons long (stearate) have been noted in human brain. Differentiation between lipases and esterases is difficult due to the overlapping specificities of the two groups (Mounter and Mounter, 1962). The very low specificity exhibited by the maize esterase isoenzymes in the present study make classifications into esterase and lipases impossible. The fact that scutellum tissue was the only tissue demonstrating 'lipase' activity may simply be due to quantitative differences in the isoenzymes between the two tissues studied. The scutellum is a tissue that stores appreciable amounts of lipid, however, and the esterases present in this tissue, especially isoenzymes 18, 34, 40, 42, and 43, may be involved in the metabolism of these lipids.

The fact that several of the major isoenzymes were capable of hydrolyzing the choline ester does not necessarily indicate that these isoenzymes are cholinesterases. As will be pointed out below, inhibition of these isoenzymes by eserine (an index used to differentiate between cholinesterases and other esterases) was not noted. The ability to hydrolyze the cholinester in the present study does indicate a very low specificity by these isoenzymes.

An esterase staining solution was made which contained equal concentrations of alpha and beta-naphthyl acetate (0.03% each). The gels containing the selected inbreds used in the substrate specificity study above were stained in this mixed substrate solution.

The beta form of the substrate resulted in red staining isoenzymes while the alpha form resulted in blue-black staining isoenzymes. By mixing the two substrates together it was hoped that a preference by the isoenzymes toward either of the two substrates could be determined by noting the color of the stained isoenzymes. It was found that isoenzyme 18 stained red in the mixed substrate solution indicating that this isoenzyme showed a preference for the beta form. The other isoenzymes stained purple to blue indicating that both substrates were being hydrolyzed and that a preference for one substrate form or the other was lacking.

5. Inhibition and Activation

The capacity to inhibit or activate the maize esterase isoenzymes was tested for approximately 40 organic and inorganic compounds. A normal staining solution, described earlier, was prepared and divided into two lots. Following electrophoresis, gels were cut into identical halves and one half was placed in the normal staining solution (control gel) and incubated at 37°C. To the remaining staining solution was added the test compound. The test compound was mixed in the normal stain and the pH of the mixture was tested and, when necessary, adjusted to pH 6.5 with either N KOH or concentrated HCl. The other half of the gel was incubated at 37°C in the test solution. Incubation times for the control gel and the test gel were the same. Dilution of the

test solution due to addition of test compound and adjustment of pH never exceeded 5%.

Inhibition and activation was rated by visual inspection of the gel by comparing the control gel with the test gel. Stainability was rated on a 0-3 scale: 0 = no stain, 1 = weak stain, 2 = normal stain equaling the control gel, and 3 = darker staining than the control gel.

Sixteen inorganic compounds were tested as inhibitors or activators of the maize esterases (Table 5). Of the 16 test compounds, three exhibited specific inhibition of certain esterase isoenzymes. Activation was not noted.

Sodium fluoride: Fluoride ion specifically inhibited isoenzyme 18. At a concentration of 1000 ppm NaF, isoenzyme 18 appeared as a weakly staining band, while it was inhibited completely at 5000 ppm. None of the other esterase isoenzymes were affected.

It has been shown that cholinesterase (Cimasoni, 1966; Heilbronn, 1965; Pastor and Fennell, 1959), arylesterases (Komma, 1963) and possibly aliesterases (Bolkova et al., 1960) are inhibited by fluoride. Furthermore, Cimasoni (1966) and Heilbronn (1965) showed that this inhibition was reversible. In the present study, a gel which showed inhibition of isoenzyme 18 following treatment with 5000 ppm NaF was soaked for one hour in running water and restained in a normal staining solution free of fluoride ion. Reactivation of isoenzyme 18, as indicated by

Table 5. The Effects of Various Inorganic Compounds on the Esterase Isoenzymes of Maize

Compound	Conc. (mg/1)	Isoenzymes									
		12	14-16	18	Complex-I	Complex-II	34	40	42	43	E_4
Sodium fluoride	1000	2*	2	1	2	2	2	2	2	2	2
	5000	2	2	0	2	2	2	2	2	2	2
Potassium permanganate	5000	2	2	2	2	2	1	1	2	2	2
Phosphate	0.1 M	2	2	2	2	2	2	2	2	2	2
	0.5 M	2	2	1	2	2	2	2	2	2	2
Potassium metaperiodate	10000	0	0	0	0	0	0	0	0	0	0
Copper chloride	5000	1	1	1	1	1	1	1	1	1	0
Copper nitrate	10000	0	0	0	0	0	0	0	0	0	0
Ferric chloride	5000	2	2	2	2	2	2	2	2	2	2
Mercuric chloride	1000	1	1	1	1	1	1	1	1	1	2
Sodium cyanide	1000	2	2	2	2	2	2	2	2	2	2
Sodium arsenate	1000	2	2	2	2	2	2	2	2	2	2
Lead acetate	1000	2	2	2	2	2	2	2	2	2	2
Stannous chloride	1000	2	2	2	2	2	2	2	2	2	2
Ferrous ammonium sulfate	5000	1	1	1	1	1	1	1	1	1	1
Ferricyanide	5000	1	1	1	1	1	1	1	1	1	1
Ferrocyanide	5000	1	1	1	1	1	1	1	1	1	1
Calcium chloride	5000	2	2	2	2	2	2	2	2	2	2

*0 = No stain (complete inhibition), 1 = weak stain (limited inhibition), 2 = normal stain (no inhibition).

staining of this isoenzyme to nearly the same intensity as the control stained gel, indicated the reversibility of fluoride inhibition.

Permanganate (as $KMnO_4$): The permanganate ion appeared to be a specific inhibitor of isoenzymes 34 and 40. At a concentration of 5000 ppm $KMnO_4$, these two isoenzymes were noticeably weakened in their stainability while the other esterase isoenzymes remained unchanged.

Phosphate: The phosphate ion appeared to specifically inhibit isoenzyme 18. The esterase staining solution used in the present study contained phosphate buffer (0.1M sodium phosphates mono- and dibasic). When the concentration of phosphate buffer was raised to 0.5M, a weakening of isoenzyme 18 was noted. Inhibition was not complete at this concentration, nevertheless a much reduced stainability of this isoenzyme was apparent. Cholinesterases have been reported to be inhibited by the phosphate ion (Cimasoni, 1966).

The other inorganic compounds tested showed no specific inhibition of select isoenzymes. Inhibition, when present, was general (Table 5).

As with the inorganic compounds tested, several of the organic compounds showed selective inhibition or activation of specific maize esterase isoenzymes (Table 6).

Eserine: Cholinesterases are inhibited at concentrations of eserine (10^{-3}M) that do not inhibit other esterases. In the

Table 6. The Effects of Various Organic Compounds on the Esterase Isoenzymes of Maize

Compound	Conc. (M)	Isoenzymes									
		12	14-16	18	Complex-I	Complex-II	34	40	42	43	E_4
Eserine sulfate	1×10^{-2}	2*	2	2	2	2	2	2	2	2	2
Atropine sulfate	2×10^{-3}	2	2	2	2	2	3	3	2	2	2
Atropine (alkaloid)	1000 ppm	2	2	2	2	2	3	3	2	2	2
Tropine	4×10^{-2}	2	2	2	2	2	2	2	2	2	2
EDTA	1×10^{-3}	2	1	2	1	1	2	2	2	2	2
	1×10^{-1}	0	0	0	0	0	0	0	0	0	0
pCMB	2.5×10^{-2}	2	0	2	0	0	0	0	2	2	2
Iodoacetamide	4×10^{-2}	2	2	2	2	2	2	2	2	2	2
Quinine sulfate	7×10^{-3}	2	2	2	2	2	2	2	2	2	2
Acetylcholine	5×10^{-3}	2	2	2	2	2	2	2	2	2	2
Cysteine	2×10^{-3}	0	0	1	0	0	1	1	1	1	1
	8×10^{-3}	0	0	0	0	0	0	0	0	0	0
Cholic acid	1×10^{-3}	2	2	2	2	2	2	2	2	2	2
Taurocholate	1×10^{-3}	2	2	2	2	2	2	2	2	2	2
Lauryl sulfate	1×10^{-3}	2	2	2	2	2	2	2	2	2	2
Tannic acid	3×10^{-2}	0	0	0	0	0	0	0	0	0	0
Thiourea	4×10^{-1}	1	1	1	1	1	1	1	1	1	1
Formamide	1×10^{0}	2	2	2	2	2	2	2	2	2	2
'Panogen'	5000 ppm	2	2	2	2	2	2	2	2	2	2

*0 = no stain (complete inhibition), 1 = weak stain (limited inhibition), 2 = normal stain (no inhibition), 3 = greater than normal stain (activation).

present study eserine sulfate at concentrations of 10^{-2} M did not inhibit any of the esterase isoenzymes. Isoenzyme 18 was altered by this concentration, staining red rather than the normal blue-black associated with the stain used. None of the other isoenzymes were affected by eserine.

Atropine: 0.1% atropine (crude alkaloid form) activated isoenzymes 34 and 40. At a concentration of 0.5% these two isoenzymes stained within 5-10 minutes, whereas a comparative stain reaction in the control gel required 20-30 minutes. Purified atropine sulfate had the same effect (2×10^{-3} M). None of the other isoenzymes were affected by either the crude alkaloid form or the purified form. Marton and Kalow (1960) reported that aromatic esterases (arylesterases) were inhibited by atropine.

Tropine: This compound, closely related to the activating compound, atropine, had no affect on any of the esterase isoenzymes in maize at the concentration used (4×10^{-3} M).

Ethylenediaminetetra acetic acid (EDTA): At a concentration of 10^{-3} M, EDTA repressed the activity of isoenzymes 14-16, Complex-I and II, 34, and 40. Other esterase isoenzymes were not inhibited at this concentration, but all of the isoenzymes were completely inhibited at a concentration of 10^{-1} M EDTA. None of the isoenzymes appeared to be affected at concentrations of 10^{-4} M or less. Complete inhibition by 10^{-1} M EDTA could be overcome by soaking the inhibited gel in an aqueous solution of

$CaCl_2$ (5%) for one hour. Following the incubation of the gel in the EDTA containing stain, the gel was washed in water and then placed in the 5% $CaCl_2$ solution for one hour and re-stained in a normal staining solution. The second stain resulted in isoenzymes approximating the staining intensity on the control gel.

EDTA is a strong chelating agent and has been reported to inhibit cholinesterases, arylesterases, and aliesterases (Marton and Kalow, 1960; Erdos et al., 1960; Lundblad, 1961; Komma, 1963; Cimasoni, 1966). It was noted by Erdos et al. (1960) and Komma (1963) that low concentrations of EDTA activated esterase activity. No such activation was noted for the maize esterases. Keay and Crook (1965) reported no activation or inhibition of hog liver esterases by EDTA but did note some activation by $CaCl_2$. In the present study $CaCl_2$ was not found to activate the esterases when used alone.

p-chloromercuribenzoate (pCMB): This thiol reagent (2.5×10^{-2} m) selectively inhibited isoenzymes 14-16, complex-I and II, 34, and 40, but had no affect on the other esterase isoenzymes. pCMB has been reported to inhibit cholinesterases, arylesterases and aliesterases (Bergmann et al., 1957; Aldridge, 1953a,b; Komma, 1963; Mounter and Whittaker, 1953; Keay and Crook, 1965).

Cysteine: Cysteine proved to be a potent inhibitor of the maize esterases. At a concentration of 8×10^{-3} M, all of the

esterase isoenzymes were completely inhibited. At a concentration of 2×10^{-3} M cysteine, isoenzymes 18, 34, 40, and the E_4 esterases retained a small amount of activity while all other esterase isoenzymes were inhibited completely. Cysteine has been reported to inhibit esterases (Mounter and Whittaker, 1953; Erdos and Laswick, 1961).

Iodoacetamide: Concentrations of iodoacetamide as high as 4×10^{-2} M had no effect on the maize esterases. Iodoacetamide has been reported to activate esterases (Komma, 1963). Other workers (Mounter and Whittaker, 1953; Keay and Crook, 1965) tested iodoacetamide and found it to have no effect on the esterase activities.

Taurocholate: A concentration of 1×10^{-3} M taurocholate had no effect on the maize esterase isoenzymes. Taurocholate has been reported to both activate (Komma, 1963) and inhibit (Pastor and Fennell, 1959) esterases.

Tannic acid: Tannic acid at a concentration of 3×10^{-2} M completely inhibited all of the maize esterase isoenzymes. At a concentration of 3×10^{-3} M a generalized inhibition of all of the isoenzymes was noted. Hall (1966) reported inhibition of tomato pectinesterase by tannic acid.

The other organic compounds tested were either ineffective as inhibitors or activators or resulted in a generalized inhibition at the concentrations used (Table 6).

Four technical grade organophosphate insecticides and four technical grade carbamate insecticides were tested for their capacities to inhibit the maize esterase isoenzymes (Table 7). A great deal of literature exists describing the potency of these compounds as esterase inhibitors and is adequately reviewed by O'Brien (1966) and van Asperen (1960).

The organophosphate compounds tested were DDVP, DIBROM, FOLITHION, and PHOSPHAMIDON.

DDVP (dimethyl-2-2-dichlorovinyl phosphate): DDVP completely inhibited isoenzymes 12, 34, 40, and the E_4 esterases at a concentration of 25 ppm (in acetone). Isoenzymes 18, 42, and 43 were partially inhibited at this concentration. At a concentration of 50 ppm DDVP, only isoenzymes 14-16 and complex-I and II retained activity. These isoenzymes were unaffected even at a concentration of 100 ppm. As is pointed out below, this compound was used in the genetic analysis of complex-I and complex-II which are normally obscured by the presence of isoenzymes 18 and 34.

DIBROM (dimethyl-1,2-dibromo-2,2-dichloroethyl-phosphate): At a concentration of 50 ppm DIBROM, all isoenzymes except 18, 42, and 43 were completely inhibited. Isoenzymes 42 and 43 were noticeably weakened at this concentration and were completely inhibited at 100 ppm DIBROM. Isoenzyme 18 was unaffected at concentrations as high as 200 ppm DIBROM.

Table 7. The Effects of Several Organophosphate and Carbamate Compounds on the Esterase Isoenzymes of Maize

Compound	Conc. (ppm)	Isoenzymes									
		12	14-16	18	Complex-I	Complex-II	34	40	42	43	E_4
DDVP*	25	0**	2	1	2	2	0	0	1	1	0
	50	0	2	0	2	2	0	0	0	0	0
DIBROM	50	0	0	2	0	0	0	0	1	1	0
	100	0	0	2	0	0	0	0	0	0	0
PHOSPHAMIDON	1000	2	2	2	2	2	2	2	2	2	2
FOLITHION	1000	2	2	2	2	2	2	2	2	2	2
SEVIN	200	0	2	1	2	2	0	0	2	2	0
	400	0	2	0	2	2	0	0	2	2	0
PYRAMAT	400	2	2	2	2	2	2	2	2	2	2
PRYOLAN	400	2	2	2	2	2	2	2	2	2	2
BAYER 39007	400	2	2	2	2	2	2	2	2	2	2

*Insecticide trade names; for active compound name, see text.

**0 = No stain (complete inhibition), 1 = weak stain (limited inhibition), 2 = normal stain (no inhibition).

Folithion (0,0-dimethyl-0-(3-methyl-4-nitrophenyl)-thionophosphate): At concentrations as high as 1000 ppm, FOLITHION proved to be ineffective as an indicator of the maize esterase isoenzymes. This compound might be expected to be ineffective against the maize isoenzymes in the present in vitro study since sulfonated organophosphates are supposedly metabolized (in vivo) to their oxygen analogues before becoming potent esterase inhibitors (O'Brien, 1966).

PHOSPHAMIDON (dimethyl-2-chloro-2-diethylcarbamoyl-1-methylvinyl phosphate): Like FOLITHION, PHOSPHAMIDON showed no inhibitory capacity towards the esterase isoenzymes at concentrations as high as 1000 ppm. Like DDVP and DIBROM, this compound is a phosphoric acid prototype and not a thiophosphoric acid prototype like FOLITHION.

The carbamates used in the present study were SEVIN, PYRAMAT, PYROLAN, and BAYER 39007.

SEVIN (N-methyl-1-naphthyl carbamate): SEVIN was the only carbamate, of the four tested, which showed a capacity for inhibiting the maize esterase isoenzymes. At a concentration of 200 ppm SEVIN, all isoenzymes except 14-16, 18, complex-I and II, 42, and 43 were completely inhibited. Isoenzyme 18 was noticeably reduced in activity at this concentration and was completely inhibited at a concentration of 400 ppm, while isoenzymes 14-16, complex-I and II, 42, and 43 retained full activity.

Mostafa et al. (1966) reported that SEVIN was metabolized by a specific esterase in cotton.

PYRAMAT (2-N-propyl-4-methylpyrimidyl(6) dimethyl carbamate), PYROLAN (1-phenyl-3-methyl-5-pyrazolyldimethyl carbamate) and BAYER 39007 (o-isopropoxyphenylmethyl carbamate) were ineffective as inhibitors of the maize esterase isoenzymes at the concentrations used in the present study.

6. Classification

Esterase classification has been based traditionally on selective inhibition and substrate specificity. Hofstee (1960) divided the esterases into three major groups: (a) ester hydrolases proper, which act upon substrates in solution, (b) lipase-type hydrolases, which act upon predominantly undissolved substrates, and (c) true lipases which act upon glycerol esters (fat splitting enzymes). As was noted in the section on substrate specificity, certain esters having acyl group chain lengths in excess of 14, and which were sparingly soluble, were hydrolyzed upon prolonged incubation. This might appear to place these esterase isoenzymes in the 'lipase-type hydrolase' group, however, due to the great degree of overlapping substrate specificities of the maize esterases and the fact that these same isoenzymes act upon the shorter chain substrates at a much faster rate make this sort of distinction difficult. More probably, the esterase isoenzymes investigated in the

present study belong to the ester hydrolase proper group described by Hofstee (1960), with the reservation that several of them have the added capacity to hydrolyze longer chained substrates.

The ester hydrolase proper group is further subdivided into four subdivisions. Cholinesterases represent one of the subdivisions of this group. Cholinesterases are differentiated from the other esterases by their sensitivity towards eserine. These enzymes are inhibited by eserine at concentrations that do not inhibit the other esterases. Two types of cholinesterases exist: (a) true cholinesterase (EC 3.1.1.7)(acetylcholinesterase or e-type) demonstrates a high order of substrate specificity towards acetylcholine, while the other type, (b) psuedocholinesterase (EC 3.1.1.8)(unspecific or s-type), in addition to hydrolyzing acetylcholine, hydrolyzes a number of aromatic and aliphatic esters as well. The two types of cholinesterases can be differentiated from each other by their substrate specificities and by selective inhibition by a number of compounds (Hawkins and Gunter, 1946; Hawkins and Mendel, 1949; Koelle, 1950; Fulton and Mogey, 1954).

The remaining three subdivisions are composed of the arylesterases (EC 3.1.1.2), carboxylesterases (EC 3.1.1.1) and the acetylesterases (EC 3.1.1.6). The arylesterases act primarily upon aromatic ester substrates. They are inhibited by

pCMB but not by organophosphates. They were originally described by Aldridge (1953a,b) and were called the A-type esterases. The carboxylesterases (aliesterases) have a very low substrate specificity but act upon a large number of aliphatic esters. They are inhibited by organophosphate compounds and were first described by Aldridge (1953a,b) who called them B-type esterases. The acetylesterases act predominantly upon aromatic esters but will also hydrolyze certain other esters as well. These esterases, like the arylesterases, are not inhibited by organophosphates. They are differentiated from the aryl-esterases by not being inhibited by pCMB. They were described by Bergmann et al. (1957) who called them the C-type esterases.

Classification of the maize esterase isoenzymes based on the criteria described above is most difficult. Isoenzyme 18 behaved much like a pseudocholinesterase (Cimasoni, 1966) in that it was inhibited (reversibly) by fluoride, and phosphate. It also hydrolyzed a choline ester (β-carbonaphthoxy choline iodide). It was not, however, inhibited by eserine. Some alteration in the activity of this isoenzyme did occur in response to eserine treatment as was evidenced by the change in color of the stain reaction. Its intense staining and utilization of non-choline esters and the lack of convincing inhibition by eserine tend to rule out a pseudocholinesterase classification. Isoenzyme 18 appeared not to be an arylesterase since it was not inhibited by pCMB.

Differentiating between the carboxylesterase and acetylesterase classifications with respect to this isoenzyme was impossible since it was inhibited by the organophosphate DDVP which would make it a carboxylesterase; but, on the other hand, it was not inhibited by the other organophosphates which would make it an acetylesterase. Myers et al. (1957) tested 47 different organophosphates and carbamates in an attempt to locate a compound that would inhibit the carboxylesterases but not the cholinesterases. The search for such a compound was unsuccessful due, as they put it, to "the fact that the group of esterases designated as aliesterases (former name for carboxylesterases) include a wide variety of different types of esterases." Differential inhibition by many of the compounds tested by this group of workers was noted. The present study tends to support their contention.

If one discounts the inability of PHOSPHAMIDON and FOLITHION to inhibit the maize esterases, isoenzymes 12, 34, 40, 42, 43, and the E_4 esterases fall into the carboxylesterase category in that they were all inhibited by DDVP and DIBROM.

It is obvious that the maize esterase isoenzymes did not lend themselves to classification based on the systems used in the past. What was obvious, however, was the fact that maize contained a very heterogeneous and complex group of esterase isoenzymes.

7. Genetic Control

Isoenzymes 60 through 80 presented a unique phenotypic multiplicity. These isoenzymes were described in the section dealing with descriptions and occurrences of the esterase isoenzymes and will not be repeated here. As was noted in that section, phenotypes of this nature were described by Harris (1966). According to Harris (1966) the four functional phenotypes and the single nonfunctional, or null, phenotype were under the control of a single locus, which he designated as the E_4 locus. A direct correlation between the E_4 esterases and isoenzymes 60 through 80 of the present study cannot be drawn due to the fact that different inbred lines of maize were used in the two studies. In both studies, however, these isoenzymes were restricted to root tissue (a characteristic not attributable to any other isoenzymes found in maize in the present study). Furthermore, the migration rates of these isoenzymes were similar in both studies. The uniqueness of the phenotypes expressed by these isoenzymes, taken with their tissue specific occurrence and migration rates, strongly indicate that the E_4 esterases described by Harris (1966) are among the isoenzymes of the present study described as isoenzymes 60 through 80. On the basis of these many similarities, the 60 through 80 series of isoenzymes were designated as E_4 esterases. Whereas Harris (1966) described his functional E_4 esterases as E_4^C, E_4^D, E_4^E, and E_4^F, the E_4 esterases

in the present study were designated as E_4^{60} through E_4^{80}.

In genetic crosses involving the Hawaiian inbreds AA6 and AA8, both of which contained isoenzyme 70 (the E_4^{70} phenotype), segregation to the null, or absent, phenotype was noted in the F_2 generation and in the backcross generation involving inbred AA8. Table 8 shows the results of the crosses involving these two inbreds. As can be seen from the table, both inbreds and their F_1 hybrid contained 70. The F_2 generation segregated 13:3 (presence:absence), and the backcross involving AA8 segregated 3:1 (presence:absence) for isoenzyme 70. The backcross involving AA6 did not segregate but showed the presence of 70 in all 201 individuals.

Selections of null type plants were made from the F_2 generation and from the backcross generation involving AA8. These plants were transplanted in the field and, at maturity, were self pollinated. The progeny seed from these selfed plants were analyzed for the E_4 esterases and the results are shown in Table 9. Of the 10 null type selfings from the F_2 population, 7 were found to segregate 1:3 (presence: absence) for 70 while 3 bred true for the null phenotype. All of the null type selfings from the backcross generation involving AA8 segregated 1:3 (presence: absence) for the 70 phenotype.

An epistatic model could be used to explain these results. Figure 4 illustrates such an epistatic model in which AA6 was

Table 8. Observed E_4 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and AA8 (Haw.)

Cross	Phenotypes 70	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	200	0	200				
AA6 x AA6	200	0	200				
AA8 x AA6	50	0	50				
(AA8 x AA6) x (AA8 x AA6)	318	85	403	13:3	326:77	1.14	0.245
(AA8 x AA6) x AA8	196	66	262	3:1	196.5:65.5	0.005	0.9
(AA8 x AA6) x AA6	201	0	201				

Table 9. Observed E_4 Esterase Ratios Derived from Selfing Null Type Plants Selected from the F_2 Population (Top), and the Backcross (AA8 x AA6) x AA8 (Bottom) of Genetic Crosses Involving Inbreds AA6 (Haw.) and AA8 (Haw.)

Plant	Phenotype 70	Null	Total	Ratio	Expected	X^2	P
1	0	40	40	0:1	0:40	0	1.0
2	12	28	40	1:3	10:30	0.53	0.49
3	13	27	40	1:3	10:30	1.20	0.26
4	0	40	40	0:1	0:40	0	1.0
5	0	40	40	0:1	0:40	0	1.0
6	9	31	40	1:3	10:30	0.13	0.73
7	10	30	40	1:3	10:30	0	1.0
8	12	28	40	1:3	10:30	0.53	0.49
9	8	32	40	1:3	10:30	0.53	0.49
10	14	26	40	1:3	10:30	2.13	0.13

Plant	Phenotype 70	Null	Total	Ratio	Expected	X^2	P
1	10	30	40	1:3	10:30	0	1.0
2	13	27	40	1:3	10:30	1.20	0.26
3	9	31	40	1:3	10:30	0.13	0.73
4	6	34	40	1:3	10:30	2.13	0.13
5	10	30	40	1:3	10:30	0	1.0
6	11	29	40	1:3	10:30	0.13	0.73

F_2

	A B	A b	a B	a b
A B	AABB	AABb	AaBB	AaBb
A b	AABb	AAbb*	AaBb	Aabb*
a B	AaBB	AaBb	aaBB	aaBb
a b	AaBb	Aabb*	aaBb	aabb

*Null types

Backcross generation involving AABB

	A B	A b	a B	a b
A B	AABB	AABb	AaBB	AaBb

Backcross generation involving aabb

	A B	A b	a B	a b
a b	AaBb	Aabb*	aaBb	aabb

*Null type

Fig. 4. Two Gene Epistatic Model Illustrating a 13:3 (Presence: Absence) Ratio in the F_2 Generation and a 3:1 (Presence: Absence) Ratio in One of the Backcross Generations Where the Genotype A_bb Represents the Null Phenotype.

assigned the AABB genotype and AA8 was assigned the aabb genotype. The F_2 generation would segregate 9 A_B_:3 A_bb:3 aaB_:1 aabb. In order to explain the absence of 70 in 3/16 of the F_2 generation, either A_bb or aaB_ would have to be assigned the null phenotype. If the null phenotype is assigned as an expression of the A_bb genotype, it can be seen from Figure 4 that the null genotypes exist in two forms (Aabb and AAbb). Of the three null type plants in the F_2 generation 2 exist as Aabb while the remaining one exists as AAbb. The null type plants occurring in the backcross generation involving inbred AA8 would all be of the Aabb genotype. It would follow that upon selfing of these null plants, 2/3 of the null selections from the F_2, those having the Aabb genotype, would segregate 1:3 (presence: absence) while the remaining 1/3 from that generation, those having the AAbb genotype, would breed true for the null phenotype. All of the null type selections from the backcross generation involving AA8 (Aabb) would segregate 1:3 (presence: absence) upon selfing. As was shown in Table 9, 7/10, or roughly 2/3, of the selections from the F_2 generation did segregate 1:3 (presence:absence) for 70 while 3/10, or roughly 1/3, did breed true for the null phenotype. As is predicted by the model, all of the null type selections from the backcross generation involving inbred AA8 segregated 1:3 (presence:absence).

Following the epistatic model, one would expect that an inbred line of maize that completely lacked an E_4 phenotype would contain the AAbb genotype. Inbred C42 lacked an E_4 isoenzyme. This null type was used in crosses involving inbreds AA8 and AA6. The data from the series of crosses involving AA6 and C42 are presented in Table 10. The F_1 hybrid showed the presence of 70 while the F_2 segregated 3:1 (presence:absence). The backcross involving C42 segregated 1:1 (presence:absence) while the other backcross showed only isoenzyme 70. These ratios are in accord with the epistatic model postulated from the series of crosses involving AA8 and AA6.

Table 11 shows the results from the crosses involving inbreds AA8 and C42. The F_1 hybrids contained 70 while the F_2 segregated 3:1 (presence:absence). The backcross involving AA8 did not segregate but showed 70 in all cases while the backcross involving C42 segregated 1:1 (presence:absence). In this series of crosses, there is a drastic departure from the ratios expected from the epistatic model. According to the epistatic model, C42 should contain the AAbb genotype resulting in the null phenotype. The F_1 hybrid involving AA8 (aabb) should show the null phenotype (Aabb). As can be seen from Table 11, the F_1 hybrid contained isoenzyme 70. According to the epistatic model, the F_2 generation would segregate 1:3 (presence:absence), but in fact did not. It segregated 3:1 (presence:absence). The

Table 10. Observed E_4 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and C42 (Minn.)

Cross	Phenotype 70	Null	Total	Ratio	Expected	X^2	P
AA6 x AA6	200	0	200				
C42 x C42	0	80	80				
AA6 x C42	40	0	40				
(AA6 x C42) x (AA6 x C42)	110	30	140	3:1	105:35	0.95	0.27
(AA6 x C42) x AA6	80	0	80				
(AA6 x C42) x C42	63	57	120	1:1	60:60	0.30	0.60

Table 11. Observed E_4 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and C42 (Minn.)

Cross	Phenotype 70	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	200	0	200				
C42 x C42	0	80	80				
AA8 x C42	20	0	20				
(AA8 x C42) x (AA8 x C42)	161	59	220	3:1	165:55	0.38	0.58
(AA8 x C42) x AA8	80	0	80				
(AA8 x C42) x C42	38	42	80	1:1	40:40	0.20	0.67

backcross generation involving inbred AA8 should have segregated 1:1 (presence:absence), but in fact showed only the presence of 70 in all individuals. The backcross involving C42 should have bred true for the null phenotype, but in fact segregated 1:1 (presence:absence).

It was obvious from this series of crosses that the epistatic model, as originally postulated, could not explain the observed results.

In a classic series of studies, Schwartz (1960, 1962a,b, 1964a,b,c,d,e, 1965, 1967) described the E_1 esterases of maize. The E_1 esterases of maize involve seven allelic isoenzymes differing in migration rates. The three most thoroughly studied E_1 esterases (E_1^S, E_1^N, and E_1^F) were among the first isoenzymes described as forming hybrid enzymes (Schwartz, 1960). Hybrids between any two inbreds containing different E_1 alleles showed both parental isoenzymes and a third hybrid isoenzyme intermediate in mobility. The E_1 esterases existed as dimers. An inbred line of maize showed a single E_1 esterase isoenzyme made up of parental subunits (autodimer) while a hybrid involving two different E_1 alleles showed, in addition to the two parental autodimers, a third allodimer, or hybrid isoenzyme. Mutants of the E_1 gene that affected the regulation of enzyme production were described by Schwartz (1962b). The 'alleles' $E_1^{F'}$ and $E_1^{S'}$ specified esterase isoenzymes (FF and SS phenotypes) having the same mobility

as those specified by the 'alleles' E_1^F and E_1^S. In developing endosperm, however, the 'alleles' $E_1^{F'}$ and $E_1^{S'}$ caused a premature cessation of enzyme production. In developing endosperm of lines homozygous for the $E_1^{F'}$ 'allele', the FF phenotype was produced up until 16 days following pollination. At this stage of development there appeared to be an abrupt cessation of enzyme production and the FF phenotype disappeared (Schwartz, 1962b). Individuals homozygous for the E_1^F 'allele' showed no such cessation of the FF phenotype. Schwartz (1962b) postulated the existence of two types of alleles specifying the E_1 esterases. The 'allele' $E_1^{F'}$, which caused premature cessation of enzyme production, was termed a 'prime allele' while those 'alleles' not causing a premature cessation of enzyme production were termed 'standard alleles'.

Heterozygotes between the $E_1^{F'}$ 'prime allele' and the E_1^N 'standard allele' showed the two parental isoenzymes (FF and NN) and the third (FN) hybrid isoenzyme in developing endosperm up until the 16th day following pollination. At stages of development beyond this, however, only the NN phenotype persisted indicating that cessation of enzyme production specified by the 'prime allele' had taken place. Schwartz (1962b) theorized that 'prime allele' containing inbreds may contain functional E_1 alleles associated with some regulatory factor, which in the case of 'prime alleles' could interact in some manner to cause cessation

of enzyme production. The 'standard allele' containing inbreds also contained the same functional E_1 allele, but contained a non-interacting regulatory factor. He represented the 'prime allele' as $R^mE_1{}^F$ and the 'standard allele' as $RE_1{}^F$. R^m regulatory factor could interact with the E_1 functional allele to cause cessation of enzyme production while R regulatory factor could not interact with the E_1 functional allele to cause cessation of enzyme production.

In an attempt to determine linkage between the R factor and the E_1 allele, Schwartz (1962b) ran experiments designed to detect recombination between the R and E_1 portions of the 'alleles'. Recombinants were not found in the 2573 individuals that would have shown recombination classes. Linkage between the regulatory factor (R) and the 'structural' allele $E_1{}^F$ was therefore close.

As was shown in the present study, the epistatic model illustrated in Figure 9 cannot explain the data presented. A modification of the model, following the example set by Schwartz (1962b) involving 'prime alleles' and 'standard alleles' can be used to explain the results. Whereas Schwartz (1962b) designated his 'prime allele' as $R^mE_1{}^F$, the 'prime allele' for the $E_4{}^{70}$ in the present study was designated as $Oe_4E_4{}^{70}$. The Oe_4 portion of the 'prime allele' in the present study is analogous to the R^m designation of Schwartz (1962b) and represents the regulatory portion of the E_4 locus. It must be stressed that the Oe_4

designation in no way implies that a discrete 'operator-type' cistron is involved in the regulation of a discrete 'structural gene'. As in the case with the E_1 esterases studied by Schwartz (1962b) recombination between the regulatory factor and the functional allele was not noted. Furthermore, the Oe_4 designation does not imply that the 'prime' and 'standard' alleles represent operons as defined by Jacob and Monod (1961). The 'standard allele' for the $E_4{}^{70}$ locus was designated as $Oe_4{}^{c}E_4{}^{70}$.

The 'repressible' nature of the 'prime allele' which resulted in cessation of enzyme production as observed by Schwartz (1962b), implies interaction between the regulatory portion of the 'prime allele' and some other factor. The ratios observed in segregating populations in the present study indicated that this interacting factor segregated independently. A second and independently segregating locus was postulated and designated as the Re_4 locus. Two alleles for this locus were postulated. Allele Re_4 was postulated as representing a functional allele which, in some way, interacted with 'prime alleles' to cause cessation of enzyme production. The alternate allele, $Re_4{}^{o}$, represented a nonfunctional allele which was incapable of interacting with 'prime alleles' to cause cessation of enzyme production.

Inbred AA8 was assigned $Re_4{}^{o}/Re_4{}^{o}$, $Oe_4E_4{}^{70}/Oe_4E_4{}^{70}$. This genotype, irrespective of the presence of the 'prime allele', was capable of producing the 70 phenotype due to the homozygosity

of the nonfunctional, independently segregating, regulatory factor (Re_4^o). Inbred AA6 was assigned Re_4/Re_4, $Oe_4{}^cE_4{}^{70}/Oe_4{}^cE_4{}^{70}$. This inbred was capable of producing the 70 phenotype due to the presence of the 'standard allele'. The F_1 hybrid between AA6 and AA8, $Re_4/Re_4{}^o$, $Oe_4{}^cE_4{}^{70}/Oe_4E_4{}^{70}$, also produced the 70 phenotype due to the presence of the 'standard allele' contributed by AA6. The F_2 population involving inbreds AA6 and AA8, which segregated 13:3 (presence:absence) (Table 8), is illustrated following the present model in Figure 5. The two backcross generations from this series of crosses are illustrated in Figure 6. Both figures show expected ratios that completely agree with the observed data. It can be seen that the F_2 generation, following the model, would be expected to segregate 13:3 (presence:absence) (Figure 5). The backcross involving AA8, according to the model, would be expected to segregate 3:1 (presence:absence), while the other backcross would be expected to show only the 70 phenotype (Figure 6).

Figure 5 illustrates that the null type individuals from the F_2 population are expressed by two genotypes. Two-thirds of these null types are heterozygous at the Re_4 locus while 1/3 of the null type genotypes are homozygous for Re_4, or the functional allele. All of the null types are necessarily homozygous for the 'prime allele'. It would follow that 2/3 of the null type plants selected from the F_2 generation would, upon selfing, segregate 1:3

		Re_4 Oe_4 E_4^{70}	Re_4 $Oe_4{}^cE_4^{70}$	$Re_4{}^o$ Oe_4 E_4^{70}	$Re_4{}^o$ $Oe_4{}^cE_4^{70}$
$\frac{Re_4}{Re_4}$	$\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}
$\frac{Re_4}{Re_4}$	$\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}
$\frac{Re_4{}^o}{Re_4{}^o}$	$\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}
$\frac{Re_4{}^o}{Re_4{}^o}$	$\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}

Fig. 5. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating the F_2 Generation Involving Inbreds AA6 (Haw.) and AA8 (Haw.)*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

		Re_4 Oe_4 E_4^{70}	Re_4 $Oe_4{}^cE_4^{70}$	$Re_4{}^o$ Oe_4 E_4^{70}	$Re_4{}^o$ $Oe_4{}^cE_4^{70}$
$Re_4{}^o$	Oe_4 E_4^{70}	$\frac{Re_4}{Re_4{}^c}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}

		Re_4 Oe_4 E_4^{70}	Re_4 $Oe_4{}^cE_4^{70}$	$Re_4{}^o$ Oe_4 E_4^{70}	$Re_4{}^o$ $Oe_4{}^cE_4^{70}$
Re_4	$Oe_4{}^cE_4^{70}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4^{70}}{Oe_4{}^cE_4^{70}}$ E_4^{70}

Fig. 6. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating the Backcross Generations Involving Inbreds AA8 (Haw.) (Top) and AA6 (Haw.) (Bottom)*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

		Re_4	$Oe_4E_4^{70}$	Re_4^{o}	$Oe_4E_4^{70}$
		Re_4	$Oe_4E_4^{70}$	Re_4^{o}	$Oe_4E_4^{70}$
Re_4	$Oe_4E_4^{70}$	Re_4	$Oe_4E_4^{70}$	Re_4	$Oe_4E_4^{70}$
			NULL		NULL
		Re_4	$Oe_4E_4^{70}$	Re_4^{o}	$Oe_4E_4^{70}$
Re_4^{o}	$Oe_4E_4^{70}$	Re_4	$Oe_4E_4^{70}$	Re_4^{o}	$Oe_4E_4^{70}$
			NULL		E_4^{70}

		Re_4	$Oe_4E_4^{70}$
		Re_4	$Oe_4E_4^{70}$
Re_4	$Oe_4E_4^{70}$	Re_4	$Oe_4E_4^{70}$
			NULL

Fig. 7. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating the Segregations of Selfed Null Type Plants Derived from the F_2 Population (Top and Bottom) and the Backcross Population (Top) Involving Inbreds AA6 (Haw.) and AA8 (Haw.).*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

(presence:absence) (Figure 7, top), while 1/3 of these selections would breed true upon selfing for the null phenotype (Figure 7, bottom). Furthermore, all of the null type selections from the backcross generation involving AA8 (Figure 6), being heterozygous for the Re_4 locus, would be expected to segregate 1:3 (presence:absence) (Figure 7, top). As can be seen from Table 9, these are, in fact, the ratios produced by the null type selections from these segregating generations.

It was noted above that inbred C42 expressed the null phenotype for the E_4 esterases. According to the present model, a null phenotype is the expression of a 'prime allele' interacting with a functional Re_4 allele. One might postulate, therefore, that C42 would contain the Re_4/Re_4, $Oe_4E_4{}^N/Oe_4E_4{}^N$ genotype, where N represents some E_4 variant (60 through 80). If this were the case, hybridization of C42 and 'prime allele' containing AA8 would result in a null phenotype. As was shown in Table 11, this was not the case. The F_1 hybrid demonstrated the 70 phenotype. This led to the postulating of a null E_4 allele ($E_4{}^{null}$). Harris (1966), in his study of the E_4 esterases, had also postulated the existence of a $E_4{}^{null}$ allele. The postulating of a null E_4 allele results in five possible null type genotypes (Figure 8). Genotypes (1) through (3) could be eliminated as possible C42 genotypes due to the fact that they all contain functional Re_4 alleles and the 'prime allele' carried by AA8 would not be expressed in the F_1 hybrid

(1) $\frac{Re_4}{Re_4} \; \frac{Oe_4 \; E_4^{N}}{Oe_4 \; E_4^{N}}$ where N = 60-80

(2) $\frac{Re_4}{Re_4} \; \frac{Oe_4 \; E_4^{null}}{Oe_4 \; E_4^{null}}$

(3) $\frac{Re_4}{Re_4} \; \frac{Oe_4{}^{c}E_4^{null}}{Oe_4{}^{c}E_4^{null}}$

(4) $\frac{Re_4{}^{o}}{Re_4{}^{o}} \; \frac{Oe_4 \; E_4^{null}}{Oe_4 \; E_4^{null}}$

(5) $\frac{Re_4{}^{o}}{Re_4{}^{o}} \; \frac{Oe_4{}^{c}E_4^{null}}{Oe_4{}^{c}E_4^{null}}$

Fig. 8. The Five Possible Null Genotypes from the Regulatory Genetic Model Controlling the E_4 Esterases.

with these three genotypes. It was postulated that C42 contained either genotype (4) or (5) of Figure 8. Both of these genotypes would result in the 70 phenotype when hybridized with AA8. As was shown in Table 11, the F_2 generation involving C42 and AA8 segregated 3:1 (presence:absence) for the 70 phenotype. The backcross involving C42 in this series of crosses segregated 1:1 (presence:absence) while the backcross involving AA8 showed only the 70 phenotype.

Figure 9 illustrates the F_2 generation involving AA8 and the two possible C42 genotypes (4, 5). The top block in the figure represents the F_2 generation when C42 contains genotype (4), or a null 'prime allele'. The expected segregation would be 3:1 (presence:absence). The backcross involving AA8 would be represented by the top row of the upper block in the figure while the backcross involving C42 would be represented by the bottom row in the upper block of the figure. It can be seen that the expected ratios agree with the observed ratios (Table 11).

The lower block of Figure 9 represents the F_2 generation from the same cross. In this instance, C42 contained genotype (5) of Figure 13, or the null 'standard allele'. The figure shows that the same ratios in all generations noted for in the upper block persist in the lower block as well.

Figures 10 and 11 represent the F_2 generations from crosses involving AA6 and C42. Figure 10 shows the ratios expected

		Re_4^o Oe_4 E_4^{70}	Re_4^o Oe_4 E_4^{null}
Re_4^o	Oe_4 E_4^{70}	Re_4^o Oe_4 E_4^{70} Re_4^o Oe_4 E_4^{70} E_4^{70}	Re_4^o Oe_4 E_4^{null} Re_4^o Oe_4 E_4^{70} E_4^{70}
Re_4^o	Oe_4 E_4^{null}	Re_4^o Oe_4 E_4^{70} Re_4^o Oe_4 E_4^{null} E_4^{70}	Re_4^o Oe_4 E_4^{null} Re_4^o Oe_4 E_4^{null} E_4^{70}

		Re_4^o Oe_4 E_4^{70}	Re_4^o $Oe_4{}^cE_4^{null}$
Re_4^o	Oe_4 E_4^{70}	Re_4^o Oe_4 E_4^{70} Re_4^o Oe_4 E_4^{70} E_4^{70}	Re_4^o $Oe_4{}^cE_4^{null}$ Re_4^o Oe_4 E_4^{70} E_4^{70}
Re_4^o	$Oe_4{}^cE_4^{null}$	Re_4^o Oe_4 E_4^{70} Re_4^o $Oe_4{}^cE_4^{null}$ E_4^{70}	Re_4^o $Oe_4{}^cE_4^{null}$ Re_4^o $Oe_4{}^cE_4^{null}$ NULL

Fig. 9. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating the Segregating Generations from Crosses Involving Inbreds AA8 (Haw.) and C42 (Minn.).*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

		Re_4 $Oe_4{}^cE_4{}^{70}$	Re_4 Oe_4 $E_4{}^{null}$	$Re_4{}^o$ $Oe_4{}^cE_4{}^{70}$	$Re_4{}^o$ Oe_4 $E_4{}^{null}$
Re_4	$Oe_4{}^cE_4{}^{70}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4{}^{null}}{E_4{}^{70}}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4{}^{null}}{E_4{}^{70}}$
Re_4	$Oe_4\ E_4{}^{null}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4{}^{null}}{NULL}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4{}^{null}}{NULL}$
$Re_4{}^o$	$Oe_4{}^cE_4{}^{70}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4\ E_4{}^{null}}{E_4{}^{70}}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4{}^{null}}{E_4{}^{70}}$
$Re_4{}^o$	$Oe_4\ E_4{}^{null}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4\ E_4{}^{null}}{NULL}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{E_4{}^{70}}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4{}^{null}}{NULL}$

Fig. 10. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating Segregating Generations from Crosses Involving Inbreds AA6 (Haw.) and C42 (Minn.) in Which C42 Contains a Null 'Prime Allele'*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designation.

		$\frac{Re_4}{}$ $\frac{Oe_4{}^cE_4{}^{70}}{}$	$\frac{Re_4}{}$ $\frac{Oe_4{}^cE_4{}^{null}}{}$	$\frac{Re_4{}^o}{}$ $\frac{Oe_4{}^cE_4{}^{70}}{}$	$\frac{Re_4{}^o}{}$ $\frac{Oe_4{}^cE_4{}^{null}}{}$
$\frac{Re_4}{}$	$\frac{Oe_4{}^cE_4{}^{70}}{}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$
$\frac{Re_4}{}$	$\frac{Oe_4{}^cE_4{}^{null}}{}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{null}}$ $E_4{}^{70}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{null}}$ NULL	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{null}}$ $E_4{}^{70}$	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{null}}$ NULL
$\frac{Re_4{}^o}{}$	$\frac{Oe_4{}^cE_4{}^{70}}{}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{70}}$ $E_4{}^{70}$
$\frac{Re_4{}^o}{}$	$\frac{Oe_4{}^cE_4{}^{null}}{}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{null}}$ $E_4{}^{70}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{null}}$ NULL	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{70}}{Oe_4{}^cE_4{}^{null}}$ $E_4{}^{70}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4{}^{null}}{Oe_4{}^cE_4{}^{null}}$ NULL

Fig. 11. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating Segregating Generations from Crosses Involving Inbreds AA6 (Haw.) and C42 (Minn.) in Which C42 A Null 'Standard Allele'.*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

from this cross when C42 contained genotype (4) while Figure 11 represents the F_2 generation expected when C42 contained genotype (5). In both instances it can be seen that the expected ratios would be the same. The F_2 generation would segregate 3:1 (presence:absence). The backcross involving AA6 would not be expected to segregate (top row in each block) but show only the 70 phenotype, while the backcross to C42 would be expected to segregate 1:1 (presence:absence) (bottom row in each block). These expected ratios are in complete agreement with the observed results (Table 10). It is impossible to determine which type of null allele (prime or standard) C42 contains unless the linkage between the Oe_4 and the E_4 factors can be broken. As yet, this linkage has not been broken.

Inbred AA8, which contained the 'prime allele' of E_4^{70}, was crossed with inbred 382 which contained isoenzyme 75. The data from this series of genetic crosses are presented in Table 12. The table shows that the F_1 hybrid between these two inbreds showed only isoenzyme 75. This indicated that inbred 382 contained the functional Re_4 allele, and, insomuch as this inbred expressed isoenzyme 75, it could be postulated that 382 also contained the 'standard allele' for 75 (E_4^{75}). The F_2 generation showed four phenotypes: 70, 70 + 75, 75 and null which, according to the model (Figure 12), should segregate 1:2:10:3, respectively. The ratios presented in Table 12 agreed well with

Table 12. Observed E_4 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and 382 (Purdue)

Cross	Phenotype 70	70+75	75	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	200	0	0	0	200				
382 x 382	0	0	200	0	200				
382 x AA8	0	0	50	0	50				
(382 x AA8) x (382 x AA8)	23	34	164	41	262	1:2:10:3	16:33:164:49	4.93	0.18
(382 x AA8) x AA8	67	66	70	48	251	1:1:1:1	62.8:62.8:62.8:62.8	5.01	0.17
(382 x AA8) x 382	0	0	147	0	147				

		Re_4 Oe_4 E_4^{70}	Re_4 $Oe_4{}^cE_4^{75}$	$Re_4{}^o$ Oe_4 E_4^{70}	$Re_4{}^o$ $Oe_4{}^cE_4^{75}$
$\frac{Re_4}{Re_4}$	$\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4\ E_4^{70}}$ E_4^{75}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4\ E_4^{70}}$ E_4^{75}
$\frac{Re_4}{Re_4}$	$\frac{Oe_4{}^cE_4^{75}}{Oe_4{}^cE_4^{75}}$	$\frac{Re_4}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{75}}$ E_4^{75}	$\frac{Re_4}{Re_4}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4{}^cE_4^{75}}$ E_4^{75}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{75}}$ E_4^{75}	$\frac{Re_4{}^o}{Re_4}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4{}^cE_4^{75}}$ E_4^{75}
$\frac{Re_4}{Re_4{}^o}$	$\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ NULL	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4\ E_4^{70}}$ E_4^{75}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4\ E_4^{70}}$ E_4^{70}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4\ E_4^{70}}$ $E_4^{70}+E_4^{75}$
$\frac{Re_4}{Re_4{}^o}$	$\frac{Oe_4{}^cE_4^{75}}{Oe_4{}^cE_4^{75}}$	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{75}}$ E_4^{75}	$\frac{Re_4}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4{}^cE_4^{75}}$ E_4^{75}	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4\ E_4^{70}}{Oe_4{}^cE_4^{75}}$ $E_4^{70}+E_4^{75}$	$\frac{Re_4{}^o}{Re_4{}^o}$ $\frac{Oe_4{}^cE_4^{75}}{Oe_4{}^cE_4^{75}}$ E_4^{75}

Fig. 12. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating the F_2 Generation Involving Inbreds AA8 (Haw.) and 382 (Purdue).*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

the expected ratios. The backcross involving AA8 was observed to segregate 1:1:1:1 for the four phenotypes, and, as can be seen from the model presented in Figure 13, this was the ratio expected for this generation. Likewise, the presence of only isoenzyme 75 in the backcross generation involving 382 (Table 12) was the ratio expected for this generation (Figure 13).

The next series of crosses involved inbreds AA6 and AA8 with inbred M119, which like inbreds AA6 and AA8, contained isoenzyme 70. The data from the series of crosses involving AA6 and M119 are presented in Table 13. The table shows that the F_1 generation contained the 70 phenotype. The segregating generations showed only isoenzyme 70 in all individuals indicating that M119, like AA6, contained the 'standard allele' for isoenzyme 70. This series of crosses did not, however, determine the allele contained at the Re_4 locus.

The series of crosses involving AA8 and M119 led to a classification of the Re_4 allele of inbred M119 (Table 14). The F_1 generation contained isoenzyme 70 while the F_2 generation segregated 13:3 (presence:absence). The backcross involving AA8 segregated 3:1 (presence:absence) while the backcross involving M119 showed only isoenzyme 70. It was indicated, therefore, that inbred M119 contained the same E_4 genotype as inbred AA6.

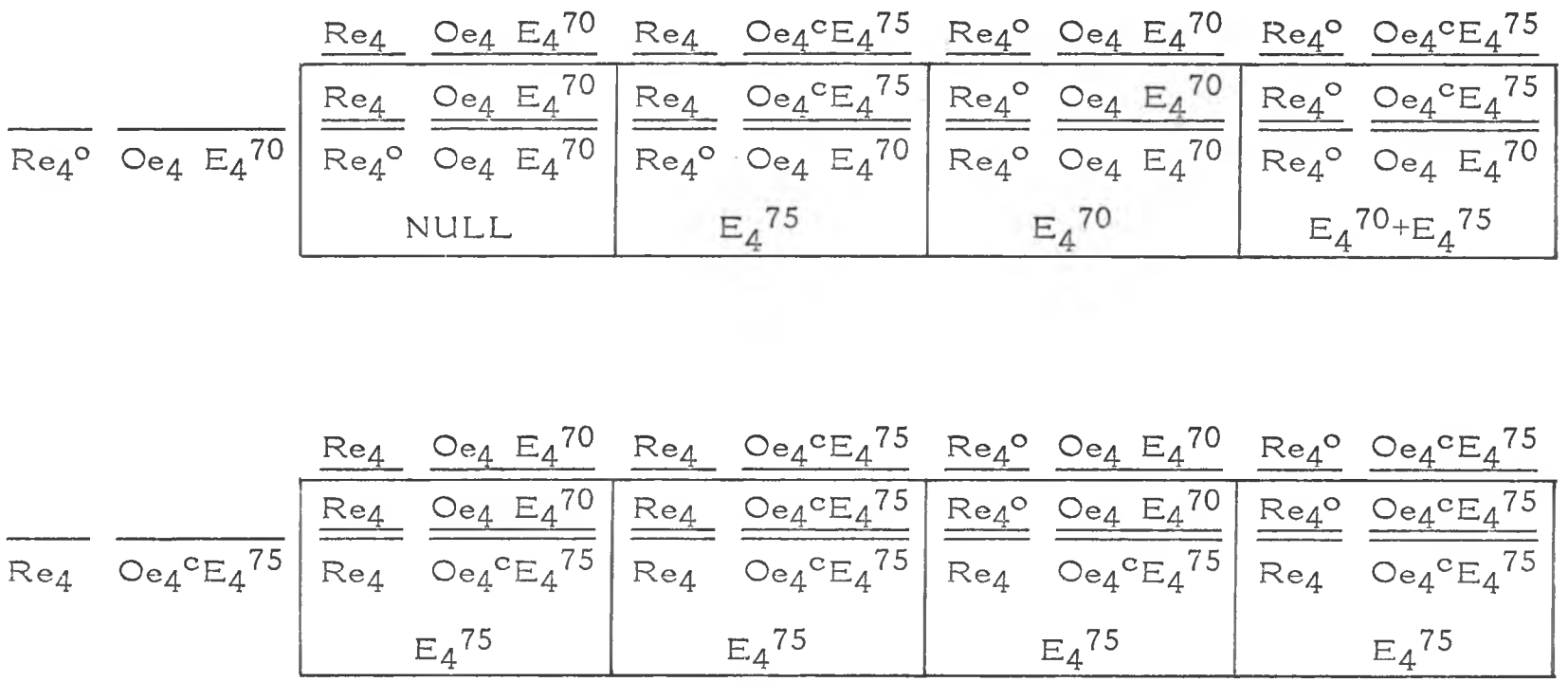

Fig. 13. Regulatory Genetic Model Controlling the E_4 Esterases and Illustrating the Backcross Generations Involving Inbreds AA8 (Haw.) (Top) and 382 (Purdue) (Bottom).*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

Table 13. Observed E_4 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and M119 (Purdue)

Cross	Phenotype 70	Null	Total
AA6 x AA6	200	0	200
M119 x M119	50	0	50
AA6 x M119	50	0	50
(AA6 x M119) x (AA6 x M119)	100	0	100
(AA6 x M119) x AA6	80	0	80
(AA6 x M119) x M119	80	0	80

Table 14. Observed E_4 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and M119 (Purdue)

Cross	Phenotype 70	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	200	0	200				
M119 x M119	50	0	50				
M119 x AA8	50	0	50				
(M119 x AA8) x (M119 x AA8)	99	25	124	13:3	100.6:23.4	0.21	0.66
(M119 x AA8) x AA8	58	22	80	3:1	60:20	0.26	0.59
(M119 x AA8) x M119	80	0	80				

Summarizing then, the E_4 esterases of maize are controlled by two independently segregating, interacting loci. 'Prime alleles', as postulated by Schwartz (1962b) and designated as $Oe_4E_4{}^N$ in the present study, interact with an independently segregating regulatory locus designated as Re_4. The Re_4 allele of the 'regulatory' locus interacts with the 'prime allele' causing cessation of enzyme production. In addition to the 'prime allele', 'standard alleles' as described by Schwartz (1962b) and designated as $Oe_4{}^cE_4{}^n$ in the present study, also exist. The 'standard alleles' do not interact with the independent regulatory locus. The regulatory locus, in addition to containing the interacting Re_4 allele, also contains an allele ($Re_4{}^o$) which is incapable of interacting with 'prime alleles' to cause cessation of enzyme production.

It is interesting to note the similarities between the model postulated in the present study and the model for genetic regulation postulated for microorganisms by Jacob and Monod (1961). The independently segregating regulatory locus (Re_4) of the present study would appear analogous to the 'regulator gene' postulated by Jacob and Monod (1961) if analogies were to be drawn. Direct analogies between the 'prime allele' of the present study and the repressible operon of the microbial systems cannot be drawn due to the fact that the Oe_4 and the $E_4{}^N$ portions of the 'prime allele' have not been shown to be independent cistrons. Similarly, only a limited analogy can be drawn between the 'standard allele' of the

present study and the constitutive operon of the microbial systems on the same basis. It therefore cannot and is not implied that the E_4 esterases are controlled by the interaction of regulator and operator genes. The lack of evidence substantiating the operon theory in higher diploid organisms along with the lack of data showing recombination between the Oe_4 and the $E_4{}^N$ portions of the E_4 locus necessitates the more conservative 'prime' and 'standard' allele designations.

Complex I and II were described in detail under the section entitled, Description and Occurrence. Each complex contains four isoenzymes. Two of the isoenzymes in each complex migrate in the area of isoenzyme 18 and are obscured in the presence of that isoenzyme. The other two isoenzymes from each complex migrate in the region of isoenzyme 34 and are obscured in the presence of that isoenzyme.

The inbreds chosen for the present study of complex-I and II were Hawaiian inbreds AA8 and AA6. Inbred AA8 was chosen out of necessity due to the fact that this inbred was the only line of corn found that contained complex-II. In addition to having complex-II, AA8 also had the masking isoenzyme 18 which obscures the faster pair of isoenzymes making up the complex. Inbred AA6 was chosen because, in addition to containing complex-I, it also contained isoenzyme 18. Other inbred lines containing complex-I were ruled out due to the fact that they contained either

masking isoenzyme 340 or both masking isoenzymes. It was thought best to use parents with identical masking effects since a search for a single compound inhibitory to the masking isoenzyme (but not to either complex) would be more fruitful than finding a compound (or combinations of compounds) inhibitory to both masking isoenzymes.

As it turned out, a compound was found that inhibited both masking isoenzymes but neither complex. By this time, however, the crosses involving AA8 and AA6 had already been made. The inhibition of the masking bands was achieved with the organophosphate DDVP (dimethyl-2-2-dichlorovinyl phosphate), and its activity was described in the section entitled, Inhibition and Activation.

The gels run for the present genetic studies were cut in half as described in the Methods section to produce two identical halves. Each half was stained for esterase activity but differed from one another in that one half received 100 ppm DDVP. This resulted in two identical gels from the standpoint of samples applied and system stained for, but differed from the standpoint that one exhibited all of the stainable esterases while the other exhibited only the two complex's under study and a few minor, non-interfering isoenzymes.

This duplication was thought necessary in order to establish the validity of the organophosphate method. It was found that all

samples exhibiting complex I in the DDVP treated gels corresponded with samples exhibiting the faster pair of isoenzymes in the region of isoenzyme 34 (isoenzymes 340^f and 350^f). Likewise, all samples exhibiting complex II in the DDVP treated gels exhibited the slower pair of isoenzymes 340^s and 350^s in the untreated gels. The validity of using an inhibitor such as DDVP was therefore established as representing the true isoenzyme spectrum for non-inhibited isoenzymes. The duplication process was used throughout the study, nevertheless. Fresh root tissue from 6-day old seedlings derived from inbreds AA8 and AA6, their F_1 hybrid, and the genetic crosses were tested for complex I and II. The data from this series of crosses are presented in Table 15. It can be seen from the table that inbred AA8 contained only complex-II and inbred AA6 contained only complex-I. The 100 F_1 hybrid seeds tested in this study were derived from five lots of 20 seeds each. Five inbred AA8 plants were selected as female parents and each was pollinated with pollen from different AA6 inbred plants. In no case was the pollen from a single AA6 plant used to pollinate more than one AA8 female. Twenty seeds were collected from each hybrid and used as the source for the F_1 root material.

As can be seen from Table 15, all 100 F_1 seeds exhibited complex-I. The same table shows that the F_2 population segregated 15:1 (complex-I:complex-II), the backcross to inbred AA8

Table 15. Observed E_5 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and AA8 (Haw.)

Cross	Phenotype Complex-I	Complex-II	Total	(I:II) Ratio	Expected	X^2	P
AA8 x AA8	0	100	100				
AA6 x AA6	100	0	100				
AA8 x AA6	100	0	100				
(AA8 x AA6) x (AA8 x AA6)	323	31	354	15:1	331.9:22.1	3.79	0.06
(AA8 x AA6) x AA8	141	48	189	3:1	142:47	0.02	0.99
(AA8 x AA6) x AA6	201	0	201				

segregated 3:1 (complex-I:complex-II), and the backcross to AA6 did not segregate but exhibited only complex-I.

Complex-I and II, on the basis of the present data, appear to be under the control of a system of recessive complementation. The model for this system is presented in Figures 14 and 15 along with the expected ratios for the segregating generations. It can be seen from the figures that 1/16 of the F_2 population would be expected to exhibit complex-II while the remaining 15/16 should exhibit complex-I. This is in good agreement with the observed data (Table 15). The expected ratios based on this model for the backcross generations are also in good agreement with the observed data.

On the basis of the data, two independently segregating loci were postulated (E_5-I and E_5-II). Each locus was postulated to contain two alleles (E_5-I^A, E_5-I^a and E_5-II^A, E_5-II^a). The doubly recessive genotype (E_5-I^a/E_5-I^a, E_5-II^a/E_5-II^a) was designated as representing complex-II while all other combinations represented complex-I.

Isoenzyme 12 was examined in seedling root tissue from crosses involving inbreds AA8 and M119. Inbred AA8 contained the isoenzyme while M119 did not. This isoenzyme is the fastest migrating common isoenzyme in the material studied.

The data from this series of crosses are presented in Table 16. It can be seen from the data that the F_1 hybrid exhibited this

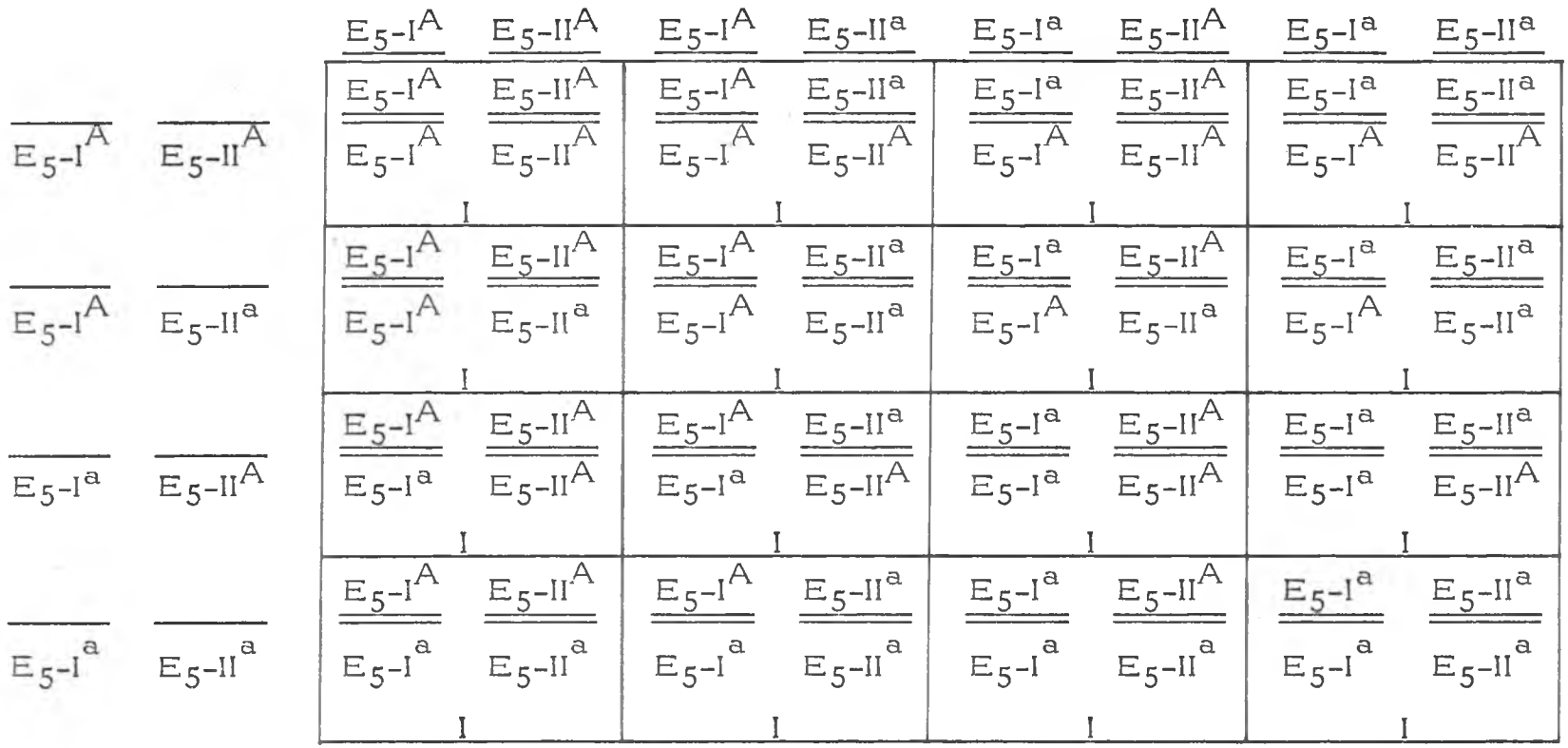

Fig. 14. Genetic Model Controlling the E_5 Esterases and Illustrating the F_2 Generation Involving Inbreds AA6 (Haw.) and AA8 (Haw.).*

*Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

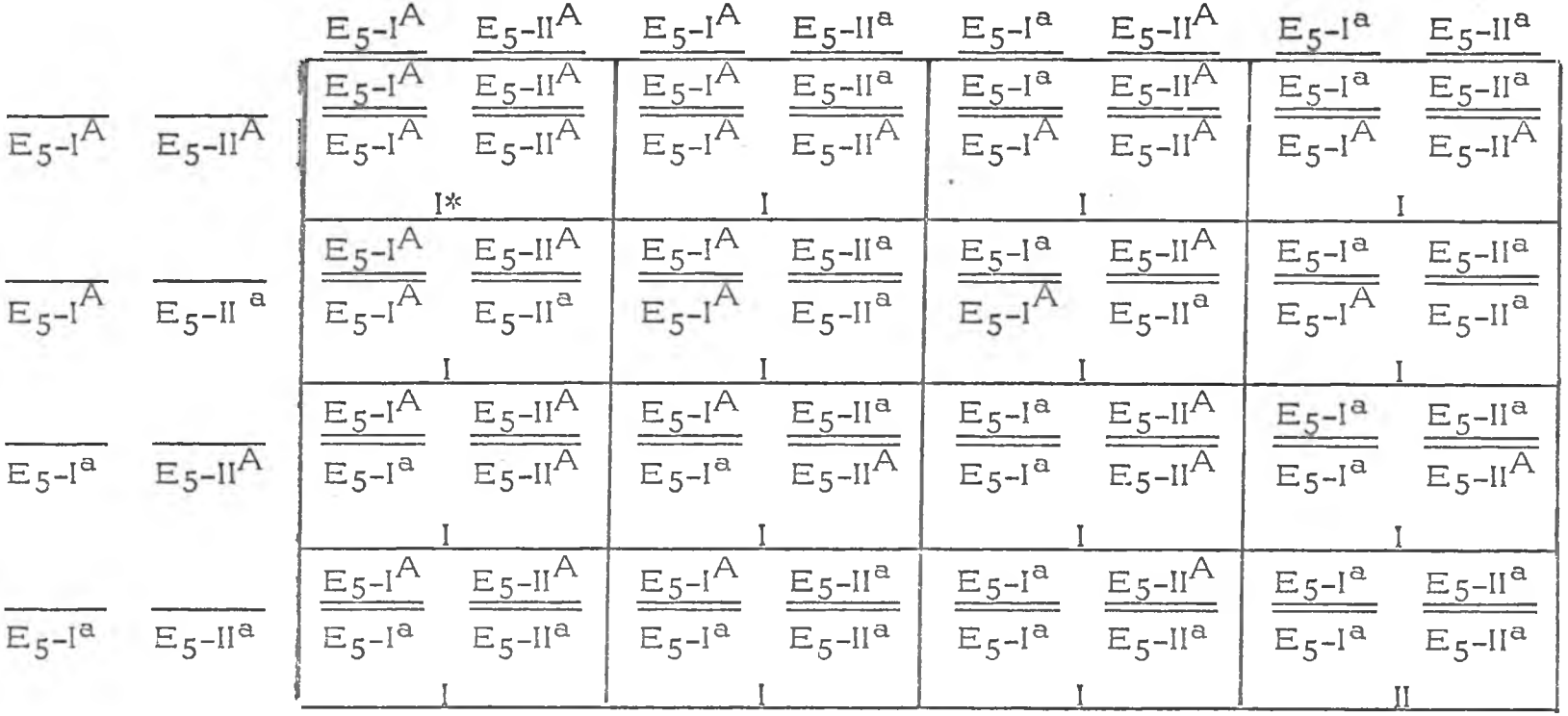

Fig. 15. Genetic Model Controlling the E_5 Esterases and Illustrating the Backcross Generations Involving Inbreds AA6 (Haw.) (Top) and AA8 (Haw.) (Bottom).**

*I = Complex-I, II = Complex-II.

**Phenotypic and genotypic designations are shown in each square. See text for inbred designations.

Table 16. Observed E_6 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and M119 (Purdue)

Cross	Phenotype 12	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	200	0	200				
M119 x M119	0	50	50				
M119 x AA8	50	0	50				
(M119 x AA8) x (M119 x AA8)	90	34	124	3:1	93:31	0.38	0.57
(M119 x AA8) x AA8	80	0	80				
(M119 x AA8) x M119	45	35	80	1:1	40:40	1.25	0.25

isoenzyme. The F_2 generation segregated 3:1 (presence: absence) and the backcross generation involving inbred M119 segregated 1:1 (presence:absence). The backcross involving AA8 did not segregate but showed the presence of this isoenzyme in all individuals.

The data indicate a simple mongenic control mechanism for the inheritance of this isoenzyme. This isoenzyme has been assigned the E_6 locus which contains two alleles (E_6^A and E_6^{null}).

Isoenzymes 14 and 16 were examined in seedling root tissue in crosses involving inbreds AA8 and AA6. Inbred AA8 lacks both of these isoenzymes while inbred AA6 contains both isoenzymes. In the present study of esterase polymorphisms, lines of maize exhibiting only one or the other of these isoenzymes were not found. In all cases, either both isoenzymes were present or both were absent.

The data from this series of crosses are presented in Table 17. It can be seen from the table that the F_1 hybrid exhibited the two isoenzymes. The F_2 generation segregated 3:1 (presence: absence) for the isoenzymes while the backcross involving inbred AA8 segregated 1:1 (presence:absence). The backcross involving inbred AA6 did not segregate but exhibited both isoenzymes in all 201 individuals.

Table 17. Observed E_7 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and AA8 (Haw.)

Cross	Phenotype 14-16	Null	Total	Ratio	Expected	X^2	P
AA6 x AA6	100	0	100				
AA8 x AA8	0	100	100				
AA8 x AA6	100	0	100				
(AA8 x AA6) x (AA8 x AA6)	174	54	228	3:1	171:57	0.20	0.68
(AA8 x AA6) x AA8	90	70	160	1:1	80:80	2.45	0.11
(AA8 x AA6) x AA6	201	0	201				

The data indicated a simple monogenic control of inheritance for the isoenzymes. In no case did either isoenzyme appear independently of the other. It appears as though both isoenzyme 14 and 16 are controlled by the same gene.

These isoenzymes have been designated the E_7 esterases. Two alleles (E_7^A and E_7^{null}) were assigned to this locus.

Isoenzyme number 18, which was found in all tissues studied, was the most prominant of the anodal esterases found in the present studies. It is unique in its behavior towards fluoride and phosphate being inhibited by these compounds whereas the other esterase isoenzymes are not. It is the only isoenzyme that is not inhibited by the organophosphate dimethyl-1,2-dibromo-2,2-dichloroethyl-phosphate (DIBROM). Isoenzyme 18 is the only isoenzyme studied here that shows a preference for beta naphthyl acetate in the presence of the alpha form. The isoenzyme is present in most inbreds of maize but is absent in C42 (Minn.), 382 (Purdue), 2253 (Iowa) and plant introductions numbered PI 181843 (Lebanon) and PI 233312 (So. Dakota).

A small amount of variation in the migration rate of isoenzyme 18 was noted in certain inbreds. The slowest phenotype was noted in a sh_2 su_2 stock received from the Maize Coop (64-643-3 selfed) while the fastest phenotype for this isoenzyme was noted in inbred 442a (Ill.). Crosses between these two variants indicated that the difference in migration was not sufficient enough

for comprehensive genetic analysis. Genetic studies were therefore limited to crosses involving the functional isoenzyme and null types. Nevertheless, due to the similarity in the unique behavior of these minor variants towards the compounds outlined above may indicate that they are alleles.

The first series of crosses in the genetic study of isoenzyme 18 involved seedling root tissue. Inbred AA8 which contained the isoenzyme and inbred 382 which lacked the isoenzyme were used as parental stocks and the data from the crosses are presented in Table 18. In Table 19 data from crosses between inbreds AA6 and C42 are presented. The data from both series of crosses indicate that isoenzyme 18 is controlled by a simple monogenic system. The data from both series show that isoenzyme was present in the F_1 hybrid, segregated 3:1 (presence:absence) in the F_2 generations, segregated 1:1 in the backcross involving the null parent, and showed only presence in the backcross involving the parent containing the isoenzyme.

Table 20 shows the results of genetic crosses involving inbreds AA5 and C42 using immature endosperm as the enzyme source. As was the case with the seedling root extracts, the endosperm data indicated a simple monogenic pattern of inheritance for isoenzyme 18.

On the basis of the data presented, a single locus was postulated (E_8). Two alleles exist (E_8^A and E_8^{null}).

Table 18. Observed E_8 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and 382 (Purdue)

Cross	Phenotype 18	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	100	0	100				
382 x 382	0	100	100				
382 x AA8	50	0	50				
(382 x AA8) x (382 x AA8)	193	69	262	3:1	196.5:65.5	0.25	0.70
(382 x AA8) x AA8	208	0	208				
(382 x AA8) x 382	77	70	147	1:1	73.5:73.5	0.33	0.60

Table 19. Observed E_8 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and C42 (Minn.)

Cross	Phenotype 18	Null	Total	Ratio	Expected	X^2	P
AA6 x AA6	50	0	50				
C42 x C42	0	50	50				
C42 x AA6	50	0	50				
(C42 x AA6) x (C42 x AA6)	96	33	129	3:1	96.8:32.2	0.003	>0.9
(C42 x AA6) x AA6	80	0	80				
(C42 x AA6) x C42	36	44	50	1:1	40:40	0.80	0.36

Table 20. Observed E_8 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and C42 (Minn.)

Cross	Phenotype 18	Null	Total	Ratio	Expected	X^2	P
AA5 x AA5	50	0	50				
C42 x C42	0	50	50				
C42 x AA5	50	0	50				
(C42 x AA5) x (C42 x AA5)	100	30	130	3:1	97.5:32.5	0.26	0.65
(C42 x AA5) x AA5	80	0	80				
(C42 x AA5) x C42	29	25	54	1:1	27:27	0.29	0.55

Homozygous E_8^A individuals show isoenzyme 18 as do heterozygotes. Homozygotes for E_8^{null} show the null phenotype.

Isoenzymes 34 and 40 were prominent isoenzymes and were found in all tissues examined in the present study. A unique characteristic of these two isoenzymes was their activation by the alkaloid atropine. The presence of this alkaloid allowed visualization of these two isoenzymes in as little time as 5 minutes. This phenomenon proved useful in allowing shorter staining times.

The first series of crosses in the genetic study of isoenzymes 34 and 40 involved inbreds AA6 and AA8 (Haw.). Inbred AA6 contained isoenzyme 40 while inbred AA8 lacked both 34 and 40 (null type). The results of the genetic crosses are presented in Table 21. It can be seen from the table that the F_1 hybrid contained isoenzyme 40. The F_2 generation segregated 3:1 (40:null). The backcross generation involving inbred AA8 segregated 1:1 (40:null) while the backcross involving AA6 did not segregate but showed only isoenzyme 40.

The second series of crosses in the present study involved inbreds AA8 and M119. M119 contained isoenzyme 34. The results of the second series of crosses are presented in Table 22. It can be seen from this table that the F_1 hybrid contained isoenzyme 34 and that the F_2 generation segregated 3:1 (34:null). The backcross generation involving AA8 segregated 1:1 (34:null) while the other backcross did not segregate but exhibited only

Table 21. Observed E_9 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and AA8 (Haw.)

Cross	Phenotype 40	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	0	200	200				
AA6 x AA6	200	0	200				
AA8 x AA6	50	0	50				
(AA8 x AA6) x (AA8 x AA6)	312	91	403	3:1	302.25:100.75	1.33	0.26
(AA8 x AA6) x AA8	129	133	262	1:1	131:131	0.06	0.43
(AA8 x AA6) x AA6	201	0	201				

Table 22. Observed E_9 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and M119 (Purdue)

Cross	Phenotype 34	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	0	200	200				
M119 x M119	80	0	80				
M119 x AA8	50	0	50				
(M119 x AA8) x (M119 x AA8)	95	29	124	3:1	93:31	0.17	0.17
(M119 x AA8) x AA8	38	42	80	1:1	40:40	0.20	0.67
(M119 x AA8) x M119	80	0	80				

isoenzyme 34.

The third series of crosses involved inbreds AA6 and M119. The data are presented in Table 23. It can be seen from this table that the F_1 hybrid contained both isoenzymes 34 and 40. The F_2 generation segregated 1:2:1 (34:34+40:40) and the backcross to AA6 segregated 1:1 (34+40:40). The backcross to M119 segregated 1:1 (34:34+40). The null phenotype was not seen.

On the basis of these results a single gene having 3 alleles was postulated. Inbred AA6 was designated as having allele $E_9{}^B$, inbred M119 was designated as having $E_9{}^A$, and inbred AA8 was designated as having allele $E_9{}^{null}$.

To further substantiate this model inbred C42 having isoenzyme 34 and, supposedly allele $E_9{}^A$, was crossed with inbreds AA8 and AA6. Table 24 presents the results from crosses involving C42 and AA6. It can be seen from this table that the F_1 hybrid contained both isoenzymes. The F_2 generation segregated 1:2:1 (34:34+40:40), the backcross involving inbred parent AA6 segregated 1:1 (34+40:40), and the backcross involving inbred parent C42 segregated 1:1 (34:34+40).

Table 25 presents the data from crosses between AA8 and C42. Here it can be seen that the F_1 hybrid contained isoenzyme 34. The F_2 generation segregated 3:1 (34:null), the backcross involving AA8 segregated 1:1 (34:null) and the backcross involving C42 did not segregate, but exhibited only 34. These

Table 23. Observed E_9 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and M119 (Purdue)

Cross	Phenotype 34	34+40	40	Total	Ratio	Expected	X^2	P
AA6 x AA6	0	0	200	200				
M119 x M119	50	0	0	80				
AA6 x M119	0	80	0	80				
(AA6 x M119) x (AA6 x M119)	107	224	109	440	1:2:1	110:220:110	0.16	0.90
(AA6 x M119) x AA6	0	54	46	100	0:1:1	0:50:50	0.64	0.73
(AA6 x M119) x M119	36	44	0	80	1:1:0	40:40:0	0.81	0.63

Table 24. Observed E_9 Esterase Ratios from Genetic Crosses Involving Inbreds AA6 (Haw.) and C42 (Minn.)

Cross	Phenotype 34	34+40	40	Total	Ratio	Expected	X^2	P
AA6 x AA6	0	0	200	200				
C42 x C42	80	0	0	80				
AA6 x C42	0	40	0	40				
(AA6 x C42) x (AA6 x C42)	30	77	33	140	1:2:1	35:70:35	1.53	0.22
(AA6 x C42) x AA6	0	42	38	80	0:1:1	0:40:40	0.20	0.90
(AA6 x C42) x C42	66	54	0	120	1:1:0	60:60:0	1.20	0.54

Table 25. Observed E_9 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and C42 (Minn.)

Cross	Phenotype 34	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	0	200	200				
C42 x C42	80	0	80				
AA8 x C42	20	0	20				
(AA8 x C42) x (AA8 x C42)	165	50	215	3:1	161.2:53.8	0.40	0.54
(AA8 x C42) x AA8	34	46	80	1:1	40:40	1.80	0.23
(AA8 x C42) x C42	80	0	80				

data, like those involving inbred M119, indicate a single locus having 3 alleles, one of which is a null type.

One further series of crosses was made. This series involved inbreds AA8 and 382. The data from this series are presented in Table 26. It can be seen from the table that the F_1 hybrid contained the 382 phenotype (34) while the F_2 segregated 3:1 (34:null). The backcross involving AA8 segregated 1:1 (34:null) while the backcross involving 382 did not segregate but exhibited only isoenzyme 34.

In all of the above series, seedling root tissue was used as the source of enzyme. In no case was an inbred found to contain both isoenzymes 34 and 40.

A limited study of these two isoenzymes was made using pollen as the tissue source (Macdonald, 1967). A total of 61 individual F_2 plants were analyzed for their 34 and 40 segregations. Inbred AA1, containing 34, and inbred AA7, containing 40, were crossed and the resulting seed planted. This hybrid was self pollinated at maturity and the resulting F_2 seed was planted. At anthesis, 61 of the F_2 plants were used as pollen sources. Pollen from the individual plants was collected and analyzed. The results are presented in Table 27.

Macdonald (1967) applied a two gene model to explain the results observed from the F_2 plants. Although null type individual samples, that would have been expected (Table 28) from the F_2

Table 26. Observed E_9 Esterase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and 382 (Purdue)

Cross	Phenotype 34	Null	Total	Ratio	Expected	X^2	P
AA8 x AA8	0	200	200				
382 x 382	200	0	200				
382 x AA8	50	0	50				
(382 x AA8) x (382 x AA8)	190	72	262	3:1	196.5:65.5	0.99	0.32
(382 x AA8) x AA8	127	124	251	1:1	125.5:125.5	0.06	0.84
(382 x AA8) x 382	147	0	147				

Table 27. Observed E_9 Esterase Ratios from Pollen Tissue Derived from 61 F_2 Plants Involving Inbreds AA1 (Haw.) and AA7 (Haw.)

Cross	Phenotype 34	34+40	40	Total	Ratio	Expected	X^2	P
AA1 x AA1	20	0	0	20				
AA7 x AA7	0	0	20	20				
AA7 x AA1	0	20	0	20				
(AA7 x AA1) x (AA7 x AA1)	11	38	12	61	1:2:1	15.25:30.5:15.25	3.72	0.13

Table 28. Expected E_9 Esterase Ratios from Pollen of 61 F_2 Plants Involving Inbreds AA1 (Haw.) and AA7 (Haw.) Following a Two Gene Model

	Phenotypes					
	34+40	34	40	Null	X^2	P
Observed	38	11	12	0	4.26	0.27
Expected	34.4	11.4	11.4	3.8		

population following a two gene model were not found, the expected F_2 ratio based on the two gene model was statistically sound. It is presently believed, however, on the basis of more extensive data from diploid tissues, that the E_9 esterases are controlled by a single locus.

Isoenzymes 50 and 57 were found only in the endosperm of maize. Of the 100 or so lines examined here, all of them contained either isoenzyme 50 or 57 but none of them contained both.

Genetic crosses were made between the two inbreds AA7 and AA1 (Haw.) in an attempt to clarify the relationship between these two isoenzymes. Inbred AA1 contained isoenzyme 57 while AA7 contained isoenzyme 50. It was found that the F_1 hybrid exhibited both isoenzymes, but two phenotypes were discernible depending on the direction of the cross. When AA7 was used as the female parent and AA1 used as the male parent, there was an unequal staining of the two isoenzymes, such that isoenzyme 50 stained approximately twice as dark as did isoenzyme 57. In the reciprocal cross the reverse was true. Isoenzyme 57 appeared to be twice as dark as isoenzyme 50.

The apparent 'dosage effect' can be explained by the triploid nature of the endosperm. The female parent contributes two doses of genetic information to the endosperm while the male contributes a single dose. One might expect, therefore, that the hybrid involving AA7, used as the female parent, would express

the maternal character (isoenzyme 50) twice as strongly as the male parent character (isoenzyme 57). The reciprocal cross would be expected to demonstrate the reverse phenotype.

The results of the genetic crosses involving these two isoenzymes are shown in Table 29. It can be seen from the table that the F_2 population segregated for four distinct phenotypes. Both parental phenotypes (50 and 57) were exhibited as well as the two phenotypes noted in F_1 reciprocal hybrids. The hybrid phenotype designated 50^m-57^w observed in the F_2 generation mimicked the phenotype of the F_1 hybrid in which AA7 was used as the female. The other hybrid phenotype of the F_2 generation designated as 50^w-57^m mimicked the reciprocal F_1 hybrid.

The four phenotypes occurred in equal frequencies in the F_2 generation, segregating 1:1:1:1. Reciprocal backcrosses were made with both parental lines. The table shows that the reciprocal backcrosses involving parent AA7 segregated 1:1 for the parental phenotype and the hybrid phenotypes. In the backcross involving AA7 as the female parent, it was found that 1:1 ratio involved parental isoenzyme 50 and the hybrid phenotype noted in AA7 x AA1 F_1 hybrids (50^m-57^w). The reciprocal backcross segregated 1:1 for the parental phenotype (50) and the phenotype noted in the reciprocal F_1 hybrid (50^w-57^m). In the reciprocal backcrosses involving inbred AA1, 1:1 ratios involving the parental phenotype (57) and the hybrid phenotypes were noted.

Table 29. Observed E_{10} Esterase Ratios from Genetic Crosses Involving Inbreds AA1 (Haw.) and AA7 (Haw.)

Cross Female x Male	Phenotypes*				Total	Ratio	Expected	X^2	P
	50^s	50^{m+57w}	50^{w+57m}	57^s					
AA1 x AA1	0	0	0	100	100				
AA7 x AA7	100	0	0	0	100				
AA1 x AA7	0	0	100	0	100				
AA7 x AA1	0	100	0	0	100				
(AA7 x AA1) x (AA7 x AA1)	62	58	66	70	256	1:1:1:1	64:64:64:64	1.25	0.7
(AA7 x AA1) x AA1	0	52	0	48	100	0:1:0:1	0:50:0:50	0.16	0.9
AA1 x (AA7 x AA1)	0	0	44	32	76	0:0:1:1	0:0:38:38	1.90	0.6
(AA7 x AA1) x AA7	75	0	67	0	142	1:0:1:0	71:0:71:0	0.45	0.9
AA7 x (AA7 x AA1)	36	42	0	0	78	1:1:0:0	39:39:0:0	0.40	0.9

*s = strong, m = medium, w = weak staining isoenzymes.

When AA1 was used as the female parent, the hybrid phenotype 50^{w}-57^{m} segregated 1:1 with isoenzyme 57. The reciprocal cross segregated 1:1 for hybrid phenotype 50^{m}-57^{w} and isoenzyme 57.

From the data presented in the table it can be seen that by pooling the hybrid phenotypes in the F_2 generation a ratio of 1:2:1 is observed. Likewise, in the backcross generations the use on non-designated hybrid phenotypes results in 1:1 ratios. The data indicated that these two isoenzymes are controlled by alleles of the same gene acting without dominance.

The frequencies of the two hybrid phenotypes in the segregating generations can be accounted for by the triploid nature of the tissue under study. Table 30 illustrates the expected ratios of all phenotypes noted in these crosses and is based on an allelic model. The table illustrates why both hybrid phenotypes are noted in reciprocal backcrosses and why the four phenotypes noted in the F_2 generation segregate 1:1:1:1.

Isoenzymes 50 and 57 were assigned the E_{10} locus. Two alleles were postulated (E_{10}^{A} and E_{10}^{B}). Homozygous E_{10}^{A} endosperm shows only isoenzyme 50. Homozygous E_{10}^{B} endosperm shows only isoenzyme 57. The heterozygote $E_{10}^{A}/E_{10}^{A}/E_{10}^{B}$ shows the $50^{s}57^{w}$ phenotype while the heterozygote $E_{10}^{A}/E_{10}^{B}/E_{10}^{B}$ shows the $50^{w}57^{s}$ phenotype.

Table 30. Expected E_{10} Esterase Ratios and Phenotypic Differences Due to the Triploid Nature of the Endosperm from Genetic Crosses Involving Inbreds AA1 (Haw.) and AA7 (Haw.) Following a One Gene Model

Cross Female x Male	Endosperm E_{10} Genotype*	Phenotypic Ratio**			
		50^{s}	$50^{m}+57^{w}$	$50^{w}+57^{m}$	57^{s}
AA1 x AA1	BBB	0	0	0	1
AA7 x AA7	AAA	1	0	0	1
AA1 x AA7	ABB	0	0	1	0
AA7 x AA1	AAB	0	1	0	0
(AA7 x AA1) x (AA7 x AA1)	AAA AAB ABB BBB	1	1	1	1
(AA7 x AA1) x AA1	AAB BBB	0	1	0	1
AA1 x (AA7 x AA1)	ABB BBB	0	0	1	1
(AA7 x AA1) x AA7	AAA ABB	1	0	1	0
AA7 x (AA7 x AA1)	AAA AAB	1	1	0	0

*AA1 = $E_{10}^{B}/E_{10}^{B}/E_{10}^{B}$; AA7 = $E_{10}^{A}/E_{10}^{A}/E_{10}^{A}$.

**s = strong, m = medium, w = weak staining isoenzymes.

L-ASPARTATE:2-OXOGLUTARATE AMINOTRANSFERASE ISOENZYMES

1. Introduction and Staining Technique

The transaminases interconvert amino acids and keto acids. The enzyme has been shown to require pyridoxal-5-phosphate as a cofactor. Generally speaking, ketoglutarate (2-oxoglutarate) serves as the universal amino acceptor in transaminase reactions. Almost any amino acid can be converted to its keto acid analogue in the presence of ketoglutarate, the cofactor, and the appropriate transaminase. The reactions are considered reversible and supposedly play an important physiological role in maintaining the appropriate amino and keto acid balance for the organism.

In the present study, the transaminase reactions observed were limited to reactions in which L-aspartate served as the amino donor. The restriction was due to the specificity of the staining technique used. Fast Violet B Salt, used in the staining technique is specific for the keto acid analogue of aspartate (oxaloacetate). Several keto acid acceptor substrates were used, ketoglutarate being found to be the most effective.

It will be shown that 13 transaminase isoenzymes exist in maize. Several were found to occur in all tissues examined. The genetic control of two transaminase isoenzymes which produce hybrid isoenzymes is reported.

Following electrophoresis the gel was cut into identical halves with a cheese cutter and one half was placed in a shallow tray with the cut surface up. The staining technique used in this study was a modification of the technique described by Decker and Rau (1963). The following stock solutions were prepared:

1) Phosphate buffer, (0.2M, pH 7.5)
2) Pyridoxal-5-phosphate, 500 µg/ml
3) Bovine albumin (Fraction 5), 3 g/100 ml
4) L-aspartic acid, 0.2M adjusted to pH 7.5 with N KOH
5) alpha-ketoglutarate (2-oxoglutaric acid), 0.1M adjusted to pH 7.5 with N KOH

The stock solutions were stored at 7°C with the exception of pyridoxal-5-phosphate which was stored frozen. Within an hour of use the following solutions were prepared:

Diazonium salt solution: Thirty mg Fast Violet B Salt (Sigma Chemical Co.) was dissolved in 1.7 ml distilled water.

Substrate solution: The following were combined and mixed thoroughly: 250 mg polyvinyl pyrrolidine (MW 40,000), 5.5 ml phosphate buffer, 0.2 ml pyridoxal-5-phosphate solution, 0.4 ml bovine albumin solution, 1.7 ml L-aspartate solution, and 0.5 ml ketoglutarate solution. The diazonium salt solution was mixed with the substrate solution and poured over the cut surface of the gel. The gel surface was kept soaked with the staining solution by collecting the solution that flowed off the surface with a

medicine dropper and re-applying it on the surface. Approximately 20 minutes at room temperature were required for incubation and resolution of the isozymes using this technique.

2. Description and Occurrence

There appeared to be approximately 13 isoenzymes of L-aspartate:2-oxoglutarate aminotransferase in the maize material studied here. The number of isoenzymes in corn contrasts with the small number of isoenzymes in animal extracts (Martinez-Carrion 1965, 1967; Decker and Rau, 1963; Kitto et al., 1967). The corn material contained only anodal transaminases while animal tissues contain both anodal and cathodal transaminases.

This section will deal with a short description of the corn transaminases and the tissue specificity of the isoenzymes. The most prominent isoenzyme found in the corn material (isoenzyme 20) has been designated as the reference isoenzyme and the migration of all of the other isoenzymes are reported in relation to the migration of this isoenzyme. Isoenzyme 20 migrates 65% of the distance of the brown borate front at pH 8.2. It has been assigned a relative mobility value of 100.

Figure 16 illustrates the isoenzymes found in maize. The figure includes the numbers assigned to the individual isoenzymes. From this figure it can be seen that the transaminases could be split up into two major groups. The faster migrating group

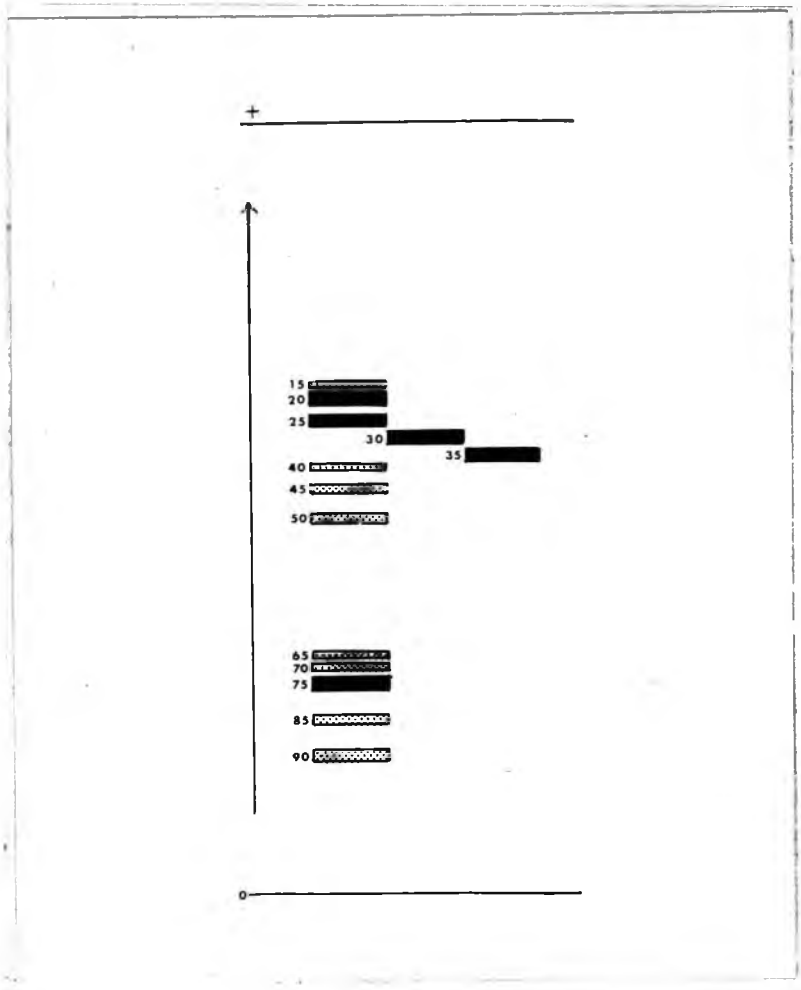

Fig. 16. An Illustration of the Transaminase Isoenzymes of Maize. The arrow indicates migration towards the anode.

included isoenzymes numbered 15-50 and had relative mobility values ranging from 102-75. The slower group of isoenzymes had relative mobility values ranging from 48-28 and were numbered from 65-90. The area between the two groups lacked any obvious transaminase activity in all tissues except mature endosperm. In mature endosperm, a small amount of transaminase activity was noted in the form of very diffuse zones. Due to the diffuseness and poorly defined nature of these zones, isoenzyme numbers were not assigned.

Isoenzyme 15, (Rm = 102), was weakly staining and is found only in plumule and root tissue from young seedlings. It was found in all inbreds tested.

Isoenzyme 20, (Rm 100), was the most prominent isoenzyme in the plant. It was found in all tissues examined and was strongest in mature leaf blades.

Isoenzymes 25, 30, and 35, (Rm 95, 92, and 89), were found in all tissues tested. These isoenzymes occurred independently of one another in inbred lines. All inbreds tested had one of the three isoenzymes.

Isoenzyme 40, (Rm 86), was found in most of the tissues examined. It was strongest in mature leaf blades and was found in all lines tested.

Isoenzyme 45, (Rm 81), was restricted to seedling plumule, root, and scutellum tissues. Most inbreds tested contained this

Table 31. Occurrence of Transaminase Isoenzymes in Different Tissues of Maize

Isoenzyme	Tissues*																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
15	w	w	0	0	0	0	w	0	0	0	0	0	0	0	0	0	0**
20	w	s	m	m	w	w	s	w	w	w	m	w	w	m	w	w	w
25	m	s	m	m	m	m	s	w	w	w	m	m	w	s	s	m	w
30	m	s	m	m	m	m	s	w	w	w	m	m	w	s	s	m	w
35	m	s	m	m	m	m	s	w	w	w	m	m	w	s	s	m	w
40	w	m	m	m	m	m	s	w	0	0	m	w	w	m	m	m	w
45	m	m	0	m	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	m	0	0	0	0	s	0	0	0	m	0	0	0	0	0	0
65	0	w	0	w	0	0	0	0	0	0	0	0	0	0	0	0	0
70	0	w	0	w	w	0	0	0	0	0	0	0	0	0	0	0	0
75	s	m	m	m	w	w	s	w	0	0	s	0	0	m	s	m	w
85	0	0	0	w	m	0	0	0	0	0	0	0	0	0	0	0	0
90	0	0	0	w	m	0	0	0	0	0	0	0	0	0	0	0	0

*1 = young root of seedling
2 = plumule
3 = coleoptile
4 = scutellum
5 = mature endosperm
6 = immature endosperm
7 = mature leaf
8 = mature root
9 = nodal area (mid plant)
10 = pith (internodal area midplant)
11 = husk
12 = young ear
13 = mature ear
14 = tassel branch
15 = mature anther
16 = pollen
17 = adventitious root

**0 = no stain, w = weak stain, m = medium stain, s = strong stain.

isoenzyme.

Isoenzyme 50, (Rm 75), was found in plumule, leaf blade, and husk tissues only. It was relatively weak and occurred in nearly all inbreds tested.

Isoenzyme 65, (Rm 48), was a relatively weak isoenzyme found only in plumule and scutellum tissues. It was present in most lines tested.

Isoenzyme 70, (Rm 46), was restricted to plumule, scutellum and endosperm of seedling tissues. It was a weak isoenzyme occurring in most of the lines studied.

Isoenzyme 75, (Rm 43), was a strong isoenzyme which occurred in most tissues. It was strongest in root tissue, anthers, mature leaf and husk.

Isoenzymes 85 and 90, (Rm 35 and 28), were found only in scutellum and endosperm of soaked seed. The dry seed did not contain these isoenzymes.

The plant transaminases have been the object of many studies over the past several decades (For reviews on the subject see Green et al., 1945; Wilson et al., 1954; Steward and Pollard, 1957; Yemm and Folkes, 1958; Virtanen, 1961; Fowden, 1967). It is generally agreed that the most common transaminase reactions involve L-glutamate and its keto acid analogue ketoglutaric acid. Apparently, glutamate serves as an amino donor for transaminations to the many keto acids reported

involved in transaminase reactions. Since the reaction is reversible, the keto acid, ketoglutarate serves as an amino acceptor for most of the amino acids. The wide scope of transamination reactions was first outlined by Meister (1967) and has been added to over the years by many authors (see reviews). The keto acids occurring widely in higher plants was tabulated by Steward and Pollard (1957) and serves as an indication of the many possible substrates for the transamination reactions.

The relationship between nitrate assimilation, the citric acid cycle, and amino acid interconversions is well established (Bonner and Varner, 1965). Nitrate, which is the chief form in which nitrogen is made available to the plant, is reduced by nitrate reductase and the particle bound nitrite reductase series. The ammonia resulting from this reduction is incorporated as amino acid nitrogen in glutamate by glutamate dehydrogenase. The citrate cycle readily supplies ketoglutarate needed for this incorporation along with other keto acids which can, in turn, interact with glutamate through transaminations to yield other amino acids. The transaminases appear to play an important physiological role by maintaining the proper amino acid balance for a given tissue through their ability to interconvert these acids.

In the present study the technique used to visualize the transaminases required that oxaloacetate be a product of the reaction. The salt used to stain the gel (Fast Violet B Salt) is specific for

this keto acid, hence aspartate constituted the amino donor in each case. The most commonly studied transaminase is glutamate: oxaloacetate transaminase (L-aspartate:2-oxoglutarate aminotransferase) or simply GOT which interconverts aspartate and glutamate and their keto acid analogues oxaloacetate and ketoglutarate. The present study involved this enzyme since ketoglutarate was used as the amino acceptor. The stain technique limits the number of transaminases that can be studied through its requirement for oxaloacetate. A stain specific for glutamate would be preferred since ketoglutarate will serve as the amino acceptor in nearly all of the transamination reactions described. Furthermore, many keto acids are not available commercially.

Generally speaking, the greatest amount of transaminase activity was found in leaf and shoot tissue. These tissues have been reported to contain most of the nitrate assimilation machinery (nitrate reductase, nitrite reductase, glutamate dehydrogenase) (Hageman et al., 1967; Beever et al., 1965; Ries et al., 1967; Ritenour et al., 1967; Tweedy and Ries, 1967; Bulen, 1956; Kessler, 1964). Other tissues not containing chlorophyll, and also microorganisms, contain the machinery for the production of glutamate from ketoglutarate and nitrate (see reviews). It is not surprising then to find transaminases in all of the tissues, nor is it surprising to find more in leaf tissue than other tissues. Nothing definite can be said about the individual isoenzymes from

a physiological sense other than that tissues containing the greatest amount of nitrate assimilatory machinery also appear to contain the greatest amount of transaminase activity. Isoenzyme 50 is unique to the leaf, husk, and plumule tissues and perhaps it plays a role in a specific interconversion of amino acids required in those tissues, but perhaps not required in other tissues. Similarly, isoenzymes peculiar to the root or some other tissue may mediate a required amino acid balance specific for that tissue.

Isoenzymes 20, 25, 30, and 35 were found to occur in all tissues examined. As will be pointed out below, isoenzyme 20 was the only one of these four isoenzymes that exhibited a specificity for ketoglutarate whereas the other three isoenzymes were able to utilize several keto acids as amino acceptors from aspartate. The fact that 3 of the four isoenzymes which exhibit no tissue specificity also have a general transaminating ability may be coincidental, but, on this basis, the transaminases can be split up into two more or less distinct classes. It appears as though, with the exception of isoenzyme 20, the nonspecific transaminases occur in all tissues while the specific transaminases, with the exception of isoenzyme 20, are restricted to certain tissues (are tissue specific). The nonspecific transaminases, which utilize several keto acids as acceptors, may play a 'general' amino acid interconversion role which is perhaps physiologically important to all tissues. On the other hand, the specific isoenzymes utilizing

only ketoglutarate as an acceptor, which are restricted to certain tissues, may mediate a highly specific transamination required in those tissues containing the isoenzymes.

3. Changes During Germination and Development

Mature seeds of several inbred lines of maize were tested at varying stages of germination to determine what changes, if any, occurred in the transaminase isoenzyme spectrum. The seed was soaked for 24 hours in running tap-water at room temperature and then transferred to petri dishes containing moistened filter paper. The petri dishes were initially maintained in the dark but were transferred to the benchtop, and light, on the third day of germination. The 24 hour soaking period constituted the first day of germination.

Tissue samples of dry seed, seed soaked for 24 hours in tap water, and seed maintained in moistened petri dishes for 24, 48, 72, 96, 124, and 144 hours were tested for transaminase activity using starch gel electrophoresis. Scutellum and endosperm tissues were tested at most of the different stages of germination. Due to the small size of the root and plumule tissues at early stages of germination, these tissues were not tested independently until the fourth day of germination (third day following transfer to the petri dishes). The entire embryo axis was tested on the third day of germination. All tissues were carefully

separated from one another and washed in distilled water prior to extraction.

Figure 17 illustrates the transaminase patterns of inbred AA6 (Haw.) at different stages of germination. It can be seen from the figure that isoenzymes 65 and 70 were noted in scutellum tissue throughout the germination period of the experiment. The embryo axis (third day of germination) lacked these isoenzymes. Isoenzyme 70 first appeared in the plumule on the fourth day of germination. It was also noted in this tissue on the fifth day of germination but disappeared again on the sixth day of germination, and was missing on the seventh day. The young root tissue failed to develop either isoenzyme 65 or 70 during the germination process.

Isoenzyme 75 was strongest in the scutellum tissue at all stages of germination. Whereas the activity of this isoenzyme appeared to maintain a steady high level in the scutellum throughout the germination process, there was a noticeable decrease in the level of activity of this isoenzyme in the plumule and root tissues with increasing age of the seedling. On the seventh day of germination the activity of this isoenzyme was very weak in the embryo axis tissues.

Isoenzymes 20 and 35 maintained a high level of activity in the scutellum throughout the germination period studied. The root tissue, on the other hand, exhibited a noticeable decrease in

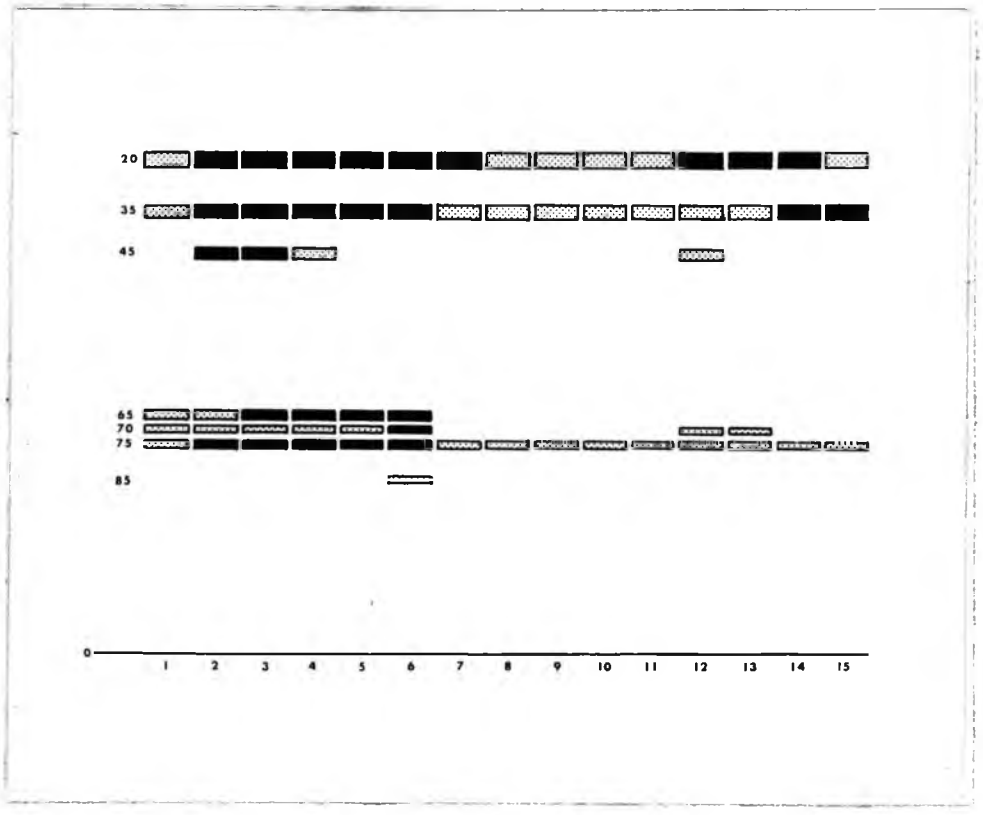

Fig. 17. Changes in the Transaminase Isoenzyme Patterns of Tissues from Germinating Seed of Inbred AA6 (Haw.) During Development of the Seedling.

*Scutellum tissue of (1) 1-day old, (2) 3-day old, (3) 4-day old, (4) 5-day old, (5) 6-day old, (6) 7-day old seedlings; (7) embryo axis of 3-day old seedling; root tissue of (8) 4-day old, (9) 5-day old, (10) 6-day old, (11) 7-day old seedlings; plumule tissue of (12) 4-day old, (13) 5-day old, (14) 6-day old, and (15) 7-day old seedlings.

the activity of these isoenzymes during the germination process.

Isoenzyme 45 was lacking in the scutellum of dry seed but exhibited strong activities in this tissue at the 3- and 4-day old stage of germination. The fifth day of germination showed an obvious decrease in activity for this isoenzyme in the scutellum and a complete lack of activity for this isoenzyme was noted in days 6 and 7. This same isoenzyme exhibited weak activity in the plumule on the third day of germination but was lacking at all other stages.

Inbred AA8 (Haw.) exhibited similar results in the tissues mentioned above over the same periods of germination but differed from inbred AA6 in the transamination spectrum of its endosperm. Figure 18 illustrates the transaminase isoenzymes of inbreds AA8 and AA6 derived from endosperm tissue grown for 5 days. It can be seen from the figure that both inbred lines exhibited a general loss of transaminase activity during germination. The endosperm of inbred AA6 lacked isoenzyme 35. There was a diffuse area of activity in the general area of isoenzyme 35 but it did not appear to be this isoenzyme due to its displacement and ill definition. Inbred AA6 contained a well defined isoenzyme 35 which lost nearly all of its activity as the seed underwent germination. It was strongest in the dry seed but dropped off sharply in activity in the germinating seed. Both inbreds exhibited a sharp decrease in activity of isoenzyme 20 with increasing age.

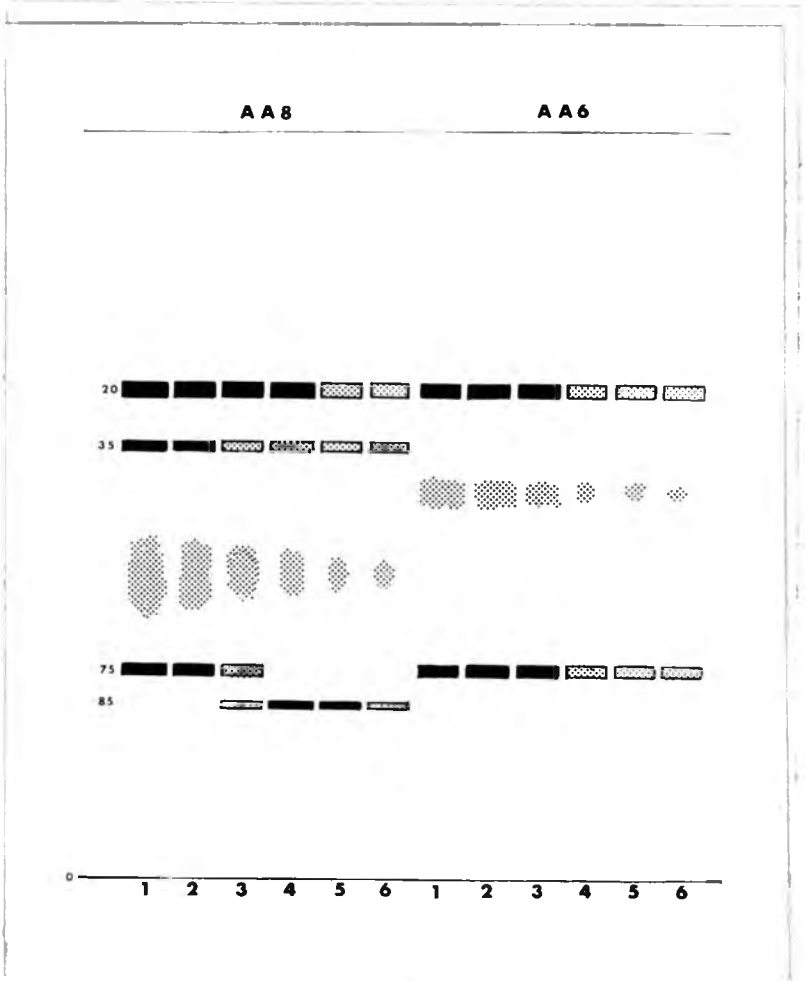

Fig. 18. Changes in the Transaminase Isoenzyme Patterns of Endosperm from Germinating Seeds of Inbreds AA8 (Haw.) and AA6 (Haw.) During Development of the Seedling.

*Endosperm from (1) dry seed and from (2) 1-day old, (3) 2-day old, (4) 3-day old, (5) 4-day old, and (6) 5-day old seedlings.

The dry seed endosperm of both inbreds exhibited the strongest amount of activity for this isoenzyme. There was a strong decrease in the activity of this isoenzyme from day one through day four and almost no activity at all on the fifth day of germination. Inbred AA6 demonstrated a loss of activity for isoenzyme 75 throughout the germination process. Inbred AA8 exhibited a complete loss of activity for this isoenzyme within the first two days of germination. The loss of activity of this isoenzyme on the third day of germination was contrasted with the appearance of isoenzyme 85. Isoenzyme 85 first exhibited activity on the second day of germination in inbred AA8 and increased in activity on the third day. The increased activity was maintained through the fourth day but dropped off sharply on the fifty day of germination.

It is apparent from the present studies that dramatic changes in the spectrum of transaminases from the different tissues of germinating seeds do occur. The significance of the changes are better understood in light of metabolic changes noted in germinating seeds of various cereals.

It was found that in germinating oat seedlings (Albaum and Cohen, 1943) transaminase activity preceded the synthesis of protein by approximately 24 hours. A sudden increase in protein synthesis beginning at about 72 hours following germination, was preceded by about 24 hours by an increase in transaminase activity and the level of soluble nitrogen. The soluble nitrogen

was presumably derived from storage protein through the action of proteases. They concluded that certain levels of soluble nitrogen and transaminase activity were required prior to the synthesis of new proteins.

During germination, there is a transfer of organic nitrogen from the endosperm to the developing embryo. In both barley (Folkes and Yemm, 1958) and pea (Danielson, 1951), specific storage proteins in the endosperm appeared to be among the first constituents of this tissue to disappear. The importance of such storage protein in the development of the embryo was illustrated by the fact that excised embryos of oat and barley (Folkes and Yemm, 1958) grow very poorly on a sugar medium. The growth of these excised embryos was increased significantly by the addition of amino acids to the growth media. This, in addition to indicating that the endosperm normally supplies amino acids, indicated that the embryo was incapable of adequately converting enough carbohydrate into the required amino acids. The fact that very little label was found in the protein produced by excised cereal embryos supplied with C-14 labelled glucose (Edelman et al., 1959) supports this contention.

Excised maize embryos, on the other hand, appear to show near normal increases in dry weight and shoot elongation when supplied with glucose (Dure, 1960a, b) and have even been grown to maturity (Andronescu, 1919). This would appear to be

an exception from the general rule that organic nitrogen is derived chiefly from the endosperm. Nason (1950), however, showed that the excised maize embryos contained abnormally low concentrations of tryptophan. This observation was expanded by Oaks and Beevers (1961a) who found that excised maize embryos contained drastically reduced levels of protein. Oaks and Beevers (1964a) went on to better define the importance of the organic nitrogen transfer from endosperm to embryo. They found that the process of polymerization of amino acids in the excised embryos to form protein was not affected per se, but that the process was limited by the reduced levels of amino acids in excised embryos. By supplying the appropriate levels of L-amino acids to the excised embryos, normal levels of protein nitrogen were approximated. It was their conclusion that the endosperm was the chief source of organic nitrogen required for normal growth of the embryo.

Cereal embryos do maintain a sizable amino acid pool as was shown by Oaks and Beevers (1964a) for corn, by Weismann (1959) for wheat and by Brown (1946) and Folkes and Yemm (1958) for barley. These pools, however, were soon depleted of their amino acids in the absence of the endosperm. The present study has shown that transamination took place in the endosperm of germinating maize kernels. The changes in the levels of transaminase activity in this tissue during the germination

process indicated different levels of amino acid interconversions at different ages of the developing seedling. The higher levels of activity in the early stages of germination as noted in the present study are in agreement with the data presented by Albaum and Cohen (1943). One might postulate that proteolytic breakdown of the storage proteins of the endosperm involves a more or less simultaneous interconversion of amino acids such that the 'balance of amino acids' and keto acids favors transportation to the embryo.

There appears to be a certain amount of debate with respect to the essential role of the endosperm in supplying organic nitrogen for the developing embryo. In the light of the experiments conducted by Brown and Morris (1890), where starch paste applied to the absorbing surface of the excised embryo of germinating barley could replace the endosperm, and the fact that Andronescu (1919) demonstrated that maize embryos (with attached scutellum) could develop into a normal mature plant when separated from the endosperm and supplied with sucrose indicated that the role of the endosperm might more properly be to supply carbohydrate.

Dure (1960a) showed that the scutellum, rather than the endosperm, served as the source of nutrient for the embryo axis in the early stages of development. Using excised embryos (scutellum intact) he noted that the scutellum lost 79% of its total nitrogen by the 12th day of germination. On the other hand, in

the intact kernel, the scutellum lost only 27% of its total nitrogen. In the intact kernel there was an initial lag period in which nitrogen was not lost from the endosperm. In fact, on the third day of germination, the nitrogen level of the endosperm had increased above the level of total nitrogen in the endosperm of the first day. It would appear, therefore, that the endosperm serves to compliment the scutellum with respect to the supply of organic nitrogen for the embryo. When present, the endosperm clearly supplies nitrogen to the embryo after an initial lag period. The scutellum would appear to serve as the initial source of nitrogen in the presence of endosperm and as the chief source of nitrogen in the absence of endosperm for the embryo.

As was noted in the present experiment, the scutellum contained the greatest amount of transaminase activity of the tissues surveyed in the germinating kernel. The dramatic changes noted in the spectrum of transaminases in the scutellum would seem to bear out the idea that the scutellum is a dynamic tissue intimately involved with the initial nutrition of the embryo and the elaboration of amino acids derived from the endosperm. The lesser amount of transaminase in the axis, especially the root tissue, would be in agreement with the idea that most of the amino acids present in the root are transported (Oaks, 1965) from other parts of the plant. The isoenzymes present in the root may be involved with amino acid interconversions required for the synthesis of specific

proteins unique to that tissue and more general metabolic pathways.

4. Substrate Utilization

In general, the transaminases of plants and animals have been shown to be specific for the L-amino acids (West et al., 1966). Exceptions to this general rule were noted in bacteria by Thorne et al. (1955). Using crude cell free extracts of Bacillus subtilis a series of transamination reactions involving D-amino acids were demonstrated. D-glutamic acid was synthesized from ketoglutaric acid and D-aspartic acid and also from ketoglutaric acid and D-alanine. The reverse reactions were demonstrated when D-aspartic acid was synthesized from D-glutamic acid and oxaloacetic acid and when D-alanine was synthesized from D-glutamic acid and pyruvic acid. Thorne and Molnar (1955) demonstrated the same reactions in similar extracts of Bacillus anthracis. Both of these bacteria contain D-glutamic acid residues in capsular polypeptides and Thorne and Molnar (1955) postulated a scheme of synthesis for this material based on the transamination reactions involving D-amino acids as amino donors.

Another exception to the general rule of L-amino acid specificity for transaminases was reported by Stumpf (1951). He reported that D-alanine served as an amino donor in reaction mixtures containing ketoglutaric acid and crude extracts from lima

bean, lupine, pumpkin, and pea seedlings as well as pumpkin leaf extracts and unheated wheat germ extracts. It was not clear, however, whether D-alanine participated directly in transamination or whether its amino group was contributed in an indirect manner.

In the present study, as was pointed out in the section on staining technique, the transamination reactions were limited to reactions which produced oxaloacetic acid as a product. This limited the study of D- and L-form amino donors to D-aspartic acid and L-aspartic acid. The staining technique described above was followed. In one case, the substrate solution contained L-aspartic acid and in the other case D-aspartic acid was used as the substrate. A single gel containing extracts from various tissues representing all of the transaminase isoenzymes previously noted was cut in half to yield two identical pieces. One piece was stained in the mixture containing L-aspartic acid as the substrate and the other piece was stained in the D-aspartate containing mixture.

None of the isoenzymes stained in the mixture containing the D-form of aspartic acid. It was concluded that the maize transaminases demonstrable by the staining technique outlined in the section on staining could not utilize the D-form of aspartic acid as an amino donor, and that these isoenzymes were specific for the L-form of aspartic acid.

A large number of amino acids undergo transamination with ketoglutaric acid. Wilson et al. (1954) demonstrated that extracts from cotyledons of lupine formed glutamic acid in mixtures involving ketoglutaric acid and 14 separate amino acids. Since then, the number of transaminase reactions using ketoglutarate as the amino group acceptor has been extended to include all of the protein amino acids and a number of non-protein amino acids (Fowden, 1967; Kretovich, 1965).

In contrast to animal and microorganism transaminases, the plant enzymes are rather poorly defined with respect to the number of different types and with regard to the keto and amino acids specificities. Several different studies have clearly indicated the existence of multiple transaminase specificities in single plants (Bone and Fowden, 1960; Leonard and Burris, 1947). The most critical data dealing with the specificity of a plant transaminase comes from the work done by Ellis and Davies (1961). Using cauliflower buds as a tissue source they purified glutamic-oxaloacetic transaminase 250 fold. They found that the enzyme could utilize cysteic and cysteinesulfinic acids as amino group donors to transaminate to either ketoglutarate or oxaloacetate. In addition to these two substrates, two compounds closely related to glutamic acid and one closely related to aspartic acid also served as amino group donors in addition to the normal substrates, glutamate and aspartate. Their studies indicated that the

cauliflower enzyme had a relatively high substrate specificity. The enzyme would not utilize pyruvate as an amino group acceptor nor would it utilize alanine as an amino group donor. They feel that this enzyme is different from the transaminase that utilizes pyruvate and alanine as substrates (glutamic-pyruvic transaminase). This is the case in animal systems (Green et al., 1945). It appeared, therefore, that the variety of transaminase reactions reported in plants were being carried out by a number of different transaminases.

In the present study an attempt was made to determine the specificity of the maize transaminases with respect to the keto acids using aspartate as the amino group donor. Root and shoot tissues from seedlings of inbreds AA8, 382 and an opaque-2 mutant line, representing inbreds containing the independently occurring isoenzymes 35, 30, and 25, respectively, were extracted and subjected to electrophoresis. Seven paired samples (root and plumule) of each inbred were applied to three separate gels such that each gel contained samples from one inbred only. Following electrophoresis, the gels were cut into identical halves and one half was stained for transaminase activity using ketoglutaric acid as the amino group acceptor and aspartic acid as the donor (normal stain). The other half of each gel was cut into seven pieces representing seven root-plumule pairs. The seven paired samples from each gel (line) were stained for transaminase

activity using aspartate as the amino group donor and seven different keto acids as acceptor substrates. The seven test keto acids were glyoxylic acid, α-ketoisocaproic acid, α-ketoisovaleric acid, β-phenylpyruvic acid, 3-indolepyruvic acid, p-hydroxyphenylpyruvic acid and pyruvic acid. These keto acids correspond to glycine, leucine, valine, phenylalanine, tryptophan, tyrosine, and alanine, respectively. The test solutions (0.1M) were adjusted to pH 7.5 with N KOH and used in place of ketoglutaric acid in individual test staining solutions. The only difference, therefore, between the normal transaminase stain described in the section on staining and the test stain solutions was the keto acid substrates.

Four of the test stain solutions gave positive results while the remaining test solutions showed no staining at all. The four keto acids that gave positive results were α-ketoisovaleric acid, β-phenylpyruvic acid, p-hydroxyphenylpyruvic acid and pyruvic acid. These keto acids correspond to valine, phenylalanine, tyrosine and alanine, respectively. In each of these test solutions each inbred showed a single isoenzyme. Both tissues exhibited the same isoenzyme. By comparison of these test gels with the corresponding samples of the gel piece stained for transaminase using the normal stain containing ketoglutarate it was possible to determine which isoenzyme appeared in the test gels. The comparison showed that the single isoenzyme exhibited in the different test solutions for a single inbred were all the same

isoenzyme. In inbred AA8 isoenzyme 35 showed activity in both tissues for all four positive test keto acids. The same specificity was noted in inbred 382 for isoenzyme 30 and for isoenzyme 25 in the opaque-2 line.

As was noted earlier, these three isoenzymes occurred in all of the tissues of maize examined in this study. Only one other isoenzyme (isoenzyme 20) was found in all tissues examined. Furthermore, each of these three isoenzymes occurred independently of one another. In the genetic study described later it will be shown that two of these three isoenzymes are under the control of a single locus (30 and 35). Genetic studies on isoenzyme 25 have not been made, but due to its similarities (occurrence in all tissues, occurrence independent of isoenzymes 30 and 35, and its lack of specificity with respect to keto acid substrates) with the other two isoenzymes leads to the speculation that this isoenzyme may also be controlled by the same locus. This lack of specificity with respect to keto acid substrates expressed by these three isoenzymes and their occurrence throughout the plant suggests that certain transaminases exist in perhaps all tissues, which are capable of carrying out a number of general transaminase reactions. This contrasts greatly with the relatively specific transaminase studied in cauliflower (Ellis and Davies, 1961) and with the other transaminases in maize which were unable to utilize any

of the test keto acids and which, with the exception of isoenzyme 20, all show tissue specificity.

5. Genetic Control

Isoenzymes 30 and 35 were found in all tissues examined in the present study. In addition, they were found to transaminate between L-aspartate and several keto acids indicating a relatively low specificity with respect to substrates. On this basis they have been termed the non-specific transaminases in the present study. In contrast to these characteristics, the other transaminases found in corn show a certain amount of tissue specificity and were found to transaminate only between L-aspartate and ketoglutarate.

The genetic study involved inbreds AA8 and 382. Inbred AA8 contained the common isoenzyme (35) while inbred 382 contained the relatively rare isoenzyme 30. Inbreds were not found that contained both of these isoenzymes.

The F_1 hybrid between these two inbreds exhibited three isoenzymes. Both parental isoenzymes were present and an additional isoenzyme intermediate in migration between the two parental types. The intermediate isoenzyme appeared to stain more intensely than either parental type in the hybrid.

The appearance of the intermediate isoenzyme can be explained on a two subunit of dimeric model. Each parental

isoenzyme is composed of two identical subunits. Isoenzyme 35 might be said to contain two B type subunits and isoenzyme 30 contains two A type subunits. The hybrid isoenzyme would then contain both types of subunits. Random combination of single subunits in the diploid tissue studied here (root) in the hybrid plant would result in the production of all three isoenzymes in the proportion 1(AA):2(AB):1(BB). This agrees well with the apparent intensities of the stains of the three isoenzymes. In each case, the intermediate isoenzyme stained more intensely than the parental isoenzymes.

The apparent dimeric nature of these isoenzymes is not surprising. An excellent study conducted by Martinez-Carrion et al. (1967) indicated that GOT is a dimer. Using pig heart as the enzyme source these authors concluded that GOT has a molecular weight of 94,000. Each subunit binds a molecule of pyridoxal-5-phosphate (cofactor) and has a molecular weight of 47,000. Their dimeric model would appear to be supported by the present study.

The results of the genetic crosses between inbreds AA8 and 382 are presented in Table 32. It can be seen from this table that the F_2 generation segregated 1:2:1 (30:hybrid:35). The backcross involving AA8 segregated 1:1 (hybrid:35) while the backcross involving 382 segregated 1:1 (30:hybrid). These results indicated that isoenzymes 30 and 35 are controlled by

Table 32. Observed Ta_1 Transaminase Ratios from Genetic Crosses Involving Inbreds AA8 (Haw.) and 382 (Purdue)

Cross	Phenotype 30	Hybrid	35	Total	Ratio	Expected	X^2	P
AA8 x AA8	0	0	20	20				
382 x 382	20	0	0	20				
382 x AA8	0	20	0	20				
(382 x AA8) x (382 x AA8)	64	132	68	264	1:2:1	66:132:66	0.12	0.92
(382 x AA8) x AA8	0	123	124	247	0:1:1	0:123.5:123.5		>0.95
(382 x AA8) x 382	78	69	0	147	1:1:0	73.5:73.5:0	0.55	0.76

alleles of the same locus. Dominance appears to be lacking and a hybrid isoenzyme is formed. The genetic model is presented in Figure 19.

The gene symbol Ta_1 was assigned to this locus and is subject to approval by the Maize Genetics Cooperation. The two alleles were designated Ta_1^A and Ta_1^B. Ta_1^A represents, when homozygous, isoenzyme 30 while the other allele, when homozygous, produces isoenzyme 35.

F_2 Generation

	Ta_1^A	Ta_1^B
Ta_1^A	$\frac{Ta_1^A}{Ta_1^A}$ 30	$\frac{Ta_1^B}{Ta_1^A}$ Hybrid
Ta_1^B	$\frac{Ta_1^A}{Ta_1^B}$ Hybrid	$\frac{Ta_1^B}{Ta_1^B}$ 35

Backcross Involving AA8

	Ta_1^A	Ta_1^B
Ta_1^A	$\frac{Ta_1^A}{Ta_1^A}$ 30	$\frac{Ta_1^B}{Ta_1^A}$ Hybrid

Backcross Involving 382

	Ta_1^A	Ta_1^B
Ta_1^B	$\frac{Ta_1^A}{Ta_1^B}$ Hybrid	$\frac{Ta_1^B}{Ta_1^B}$ 35

Fig. 19. Genetic Model Controlling the Ta_1 Transaminases and Illustrating the Segregating Generations Involving Inbreds AA8 (Haw.) and 382 (Purdue).

PHOSPHORYLASE ISOENZYMES

1. Introduction and Staining Technique

Two general techniques were used to visualize the phosphorylase isoenzymes following electrophoresis. Polyacrylamide gels were used in the study of phosphorylase due to the fact that the staining reactions involved carbohydrate metabolism. The reaction carried out by phosphorylase involves the transfer of a sugar moiety (glucose) from a cori ester (glucose-1-phosphate) to the non-reducing end of a dextrin molecule (amylose, amylopectin, glycogen, etc.):

$$\text{glucose-1-phosphate} + (\text{polyglucose})_n \longleftrightarrow (\text{polyglucose})_{n+1} + \text{Pi}$$

The reaction is reversible and the staining technique employed in the present study utilized both reactions for phosphorylase demonstration. The reaction from left to right was designated as the synthetic reaction while the reaction from right to left was designated as the degradative reaction.

Synthetic reaction stain: Following electrophoresis, the gel was incubated at 37°C in a solution composed of 100 ml Tris-citrate buffer (pH 6.2, Table 1) containing 0.25% glucose-1-phosphate. Following two hours of incubation, the incubation media was poured off and the gel was rinsed with water, then immersed in a staining solution. The staining solution consisted of

a 0.25% KI solution. Just prior to use, 100 ml of the staining solution was poured into a beaker to which had been added 0.5 ml concentrated acetic acid (glacial) and 1 ml 3% hydrogen peroxide. The hydrogen peroxide oxidized the KI to produce iodine in a slightly acidic mixture. The mixture was poured over the gel and was allowed to stand for approximately 10 minutes, or until the newly synthesized amylose-like polysaccharide had stained a bright blue in color. The blue color resulting from the starch-iodine interaction maintained its intensity providing the gel was stored in an aqueous solution containing 2% acetic acid. When the gel was stored in water alone the blue isoenzyme bands faded out completely within a few days, but could be restained with the KI mixture.

Degradative stains: The acrylamide gel was incubated at 37°C in 0.2M phosphate buffer (pH 6.2) containing 2% hydrolyzed starch for two hours. The buffer-starch solution was heated to dissolve the starch and then cooled to room temperature prior to immersion of the gel. Following incubation, the gel was washed in water and stained in the same manner as was the synthetic reaction gels. The phosphorylase isoenzymes appeared as clear zones against a blue background. The same technique also stained for amylase isoenzymes. Differentiation between the phosphorylase isoenzymes and the amylase isoenzymes was accomplished in two ways. Comparison of the degradatively stained

gels and the synthetically stained gels allowed matching of the isoenzymes. Overlaying of the degradatively stained gels with the synthetically stained gel showed that the same isoenzymes were involved in both reactions. The amylase isoenzymes did not coincide with zones producing polysaccharide in the synthetically stained gel. The second method of differentiating between phosphorylase activity and amylase activity involved incubating a gel in tris-citrate buffer (0.1M, pH 6.2) containing 2% hydrolyzed starch. This technique eliminated phosphorylase isoenzymes by eliminating the source of phosphate required by that enzyme in the degradative reaction. In this case only amylase isoenzymes produced clear regions in the gel.

A second method used in the degrative stain for phosphorylase isoenzymes involved incorporating starch into the acrylamide gel prior to electrophoresis. Hydrolyzed starch, amylose, or amylopectin (0.3%) was added to the buffers used to make up the gel and dissolved by heating. After cooling, the ingredients for gelation were added. The gel was electrophoresed in the usual manner and finally incubated in 0.2M phosphate buffer for two hours at 37°C. The gel was rinsed and stained with the KI solution. Here again, amylase activity was also noted but could be differentiated from the phosphorylase activity by incubating this type of gel in the tris-citrate buffer instead of the phosphate buffer. Likewise, this type of gel, containing incorporated

dextrins, could be used for the synthetic stain by incubating in the glucose-1-phosphate media.

2. Description and Occurrence

Phosphorylase isoenzyme activity, following the staining procedures outlined above, was examined in immature endosperm (16 days following pollination) of maize lines that were homozygous for the endosperm mutant genes su_1, su_2, o_2, du_1, ae, wx, and the normal starchy endosperm. The endosperm mutant genes, with the exception of su_1 were incorporated into a common background stock (K55). Whole mature dry seed extracts involving mutants ae, wx, aewxsu_1, and su_1, starchy inbreds Oh7 and Oh43 were also studied as were root, coleoptile, plumule, scutellum, and endosperm from seven-day old seedlings of su_1 inbred AA3. In addition to the maize material, potato tuber extracts from baking and salad type tubers were studied.

Figure 20 illustrates the phosphorylase isoenzymes found in the tissues of maize and the potato tuber material. The group of three isoenzymes of maize, designated as isoenzymes A-C in the illustration, were found in immature endosperm and whole seed extracts only. Tissues of the seven-day old seedling did not contain these isoenzymes. The maize phosphorylase isoenzyme designated as D in the figure was a diffuse band occurring in scutellum, coleoptile, and plumule only. It was not found in the

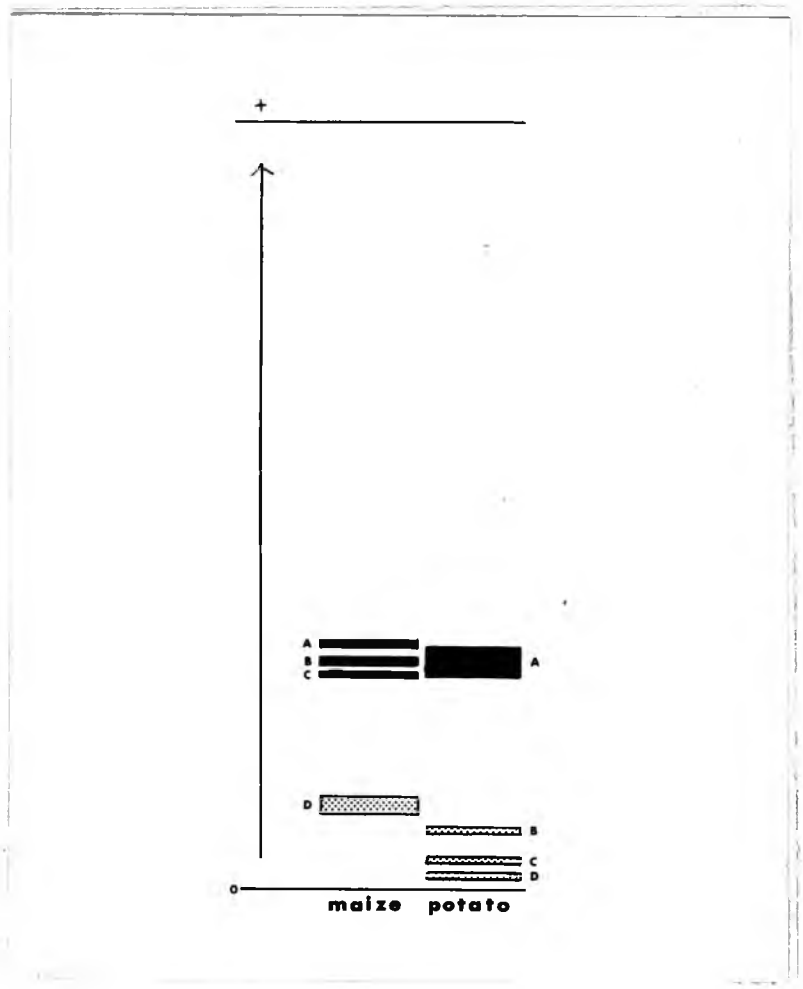

Fig. 20. An Illustration of the Phosphorylase Isoenzymes of Maize and Potato. Arrow indicates migration towards the anode.

immature endosperm, whole seed extracts, or the root and endosperm tissues of the young seedling. It appeared as though isoenzymes A-C, which occurred in the whole seed extracts from dry seeds, disappeared shortly after germination, and that isoenzyme D appeared sometime after or during germination since it was not present in the dry seeds.

The potato extracts contained 4 isoenzymes; a major isoenzyme, designated as A in the figure, that migrated to a position corresponding to that of the A-C maize isoenzymes, and three slower isoenzymes designated as B-D in the figure. When mixtures of the potato extract and immature endosperm were electrophoresed, isoenzyme A from the maize material migrated just ahead of the zone containing isoenzymes B and C of maize and A of potato, and which stained heavily as a single strong isoenzyme.

3. Primer Requirements

Glucose, maltose, hydrolyzed starch, amylose (corn), amylopectin (corn), and glycogen (shellfish) were tested as substrate primer materials or acceptor molecules for the transfer of glucose from glucose-1-phosphate in the synthetic reaction mediated by phosphorylase. One-tenth percent of these materials was incorporated into acrylamide gels. Glucose, maltose, and glycogen were readily soluble in the buffer solutions used to prepare the gels. Amylose, amylopectin, and hydrolyzed starch had

to be heated in the buffer solution in order to dissolve. One hundred mg of these materials were mixed with 100 ml of gel buffer and heated until dissolved. The mixture was cooled to room temperature and the ingredients for gelation were added. The gels were electrophoresed in the usual manner and eventually stained for the synthetic reaction.

It was found that isoenzymes A, B, and C of maize and isoenzyme A of potato were capable of synthesizing amylose-like polysaccharide in the absence of primer material. The isoenzymes of maize stained weakly in the absence of primer material but staining was evident. The addition (incorporation) of primer materials enhanced the synthetic ability of the maize isoenzymes. Glucose and maltose served as fair primer materials while the other three primers greatly enhanced the synthetic ability of the maize phosphorylases.

Isoenzyme D of maize was unable to synthesize amylose-like polysaccharide material with primers other than glycogen. Glucose was not tested as a primer material for this isoenzyme, but amylose, amylopectin, and maltose were found to be ineffective as primer, or glucose acceptor, materials.

Isoenzyme A of the potato extracts had the capacity of synthesizing amylose-like polysaccharide in the absence of primer material. Isoenzymes C and D required primer material for synthesis as did isoenzyme B. Amylose, maltose, glucose,

glycogen, and amylopectin all served as acceptor material for isoenzymes C and D while only amylose and amylopectin served as primer material for isoenzyme B. Glycogen and the two sugars were ineffective as primer molecules for this isoenzyme.

The requirement by phosphorylase for primer materials for the synthesis of amylose has been a matter of dispute for some time. The classic studies by Cori et al. (1937, 1943a,b,c) and Green and Cori (1943) indicated that primer material was required by phosphorylase for synthesis of amylose. In 1961, Illingworth et al. and Brown et al. found that muscle phosphorylase synthesized amylose in the presence of high concentrations of glucose-1-phosphate without exogenous primer material. This was questioned by Abdullah et al. (1965) who repeated the test using highly purified glucose-1-phosphate. It appeared as though the unpurified glucose-1-phosphate used in the earlier studies had supplied small amounts of primer material and that the use of highly purified glucose-1-phosphate resulted in a lack of synthetic power by muscle phosphorylase in the absence of primers. Watkins et al. (1965) noted that potato phosphorylase synthesized amylose in the absence of primer material and they concluded that potato phosphorylase contained bound carbohydrate material which could act as primer molecules. Kamogawa et al. (1968) noted the same thing using purified potato phosphorylase. The potato phosphorylase could synthesize amylose in the presence of highly

purified glucose-1-phosphate without the presence of primer. Kamogawa et al. (1968) treated their purified potato phosphorylase with glucoamylase and found that following such a treatment, the potato phosphorylase had lost its capacity to synthesize amylose in the absence of primer. They concluded that potato phosphorylase bound carbohydrate material which, when present, could act as primer material. Tsai and Nelson (1968) fractionated two types of phosphorylase (I and II) from maize endosperm using column chromatography. The phosphorylase I from this study required the presence of primer for synthesis of amylose. Phosphorylase II from this study had the capacity of synthesizing amylose in the absence of exogenously supplied primer. This phosphorylase II could use maltose as primer material while the phosphorylase I could not.

It seems likely that the 'primer-free' phosphorylases in the present study (isoenzymes A, B, C of maize and isoenzyme A of potato) may also bind carbohydrate material as was shown for potato phosphorylase (Watkins et al., 1965; Kamogawa et al., 1968). Their enhanced activities in the presence of primers agrees with the studies by these two groups.

Further indirect evidence in support of this bound carbohydrate theory was indicated following fractionation of the potato phosphorylase. Potato juice was brought to 67% saturation with ammonium sulfate and the precipitate was redissolved in a

minimum amount of distilled water. This solution was dialysed against 3 liters of distilled water for 24 hours and re-precipitated with ammonium sulfate at 55% saturation. The second precipitate was dissolved in a minimum amount of distilled water and used as a source of phosphorylase. It was found that the twice fractionated or semi-pure phosphorylase had lost most of its capacity to synthesize amylose in the absence of primers. Gels containing no primer and containing the semi-pure preparation and untreated potato juice, when stained for phosphorylase activity, showed a very weak isoenzyme A in the semi-pure sample and a relatively strong isoenzyme in the untreated potato juice. The same preparations, when electrophoresed in a gel containing glycogen as primer, showed equally active isoenzymes. This indicated that the fractionation of the potato phosphorylase had removed most of its capacity to synthesize amylose in the absence of primers. The presence of primers, however, restored the activity of the semi-pure preparation. It could be reasoned that the fractionation procedure had removed some of the bound carbohydrate, thereby removing some of the capacity of the isoenzyme to synthesize amylose in the absence of exogenously supplied primer.

AMYLOPECTIN-1,6-GLUCOSIDASE ISOENZYMES

1. Introduction and Staining Technique

Amylopectin-1,6-glucosidase (R-enzyme) was originally described by Hobson et al. (1950). The enzyme hydrolyzes 1-6 glucosidic linkages of branched dextrins most notably amylopectin (Hobson et al., 1951) and does not debranch glycogen (Peat et al., 1954). In 1958, MacWilliam reported the heterogeneity of broad bean R-enzyme. Fractionation on alumina gel yielded two fractions having R-enzyme activity. One fraction was specific for the debranching of amylopectin while the other fraction debranched only β-limit dextrin. They concluded that the activity noted by earlier workers, which showed that R-enzyme debranched both amylopectin and β-limit dextrin (Hobson et al., 1950, 1951) was due to a mixture of these two fractions. MacWilliam and Harris (1959) found that malt also contained two enzymes capable of debranching amylopectin or its degradation products. One enzyme debranched amylopectin and β-limit dextrin, while the other debranched only β-limit dextrin. They reserved the name R-enzyme for the enzyme that attacked amylopectin. The name limit dextrinase was applied to the other enzyme.

Accompanying the debranching of amylopectin is a sharp increase in the blue value. The red-purple color of amylopectin when stained with iodine is shifted to a blue color following

R-enzyme activity. The staining technique utilized this shift in color to locate the R-enzyme isoenzymes.

Two-tenths percent amylopectin (corn) was incorporated into acrylamide gels prior to electrophoresis. Following electrophoresis, the gels were incubated in 0.2 M phosphate-citrate buffer (pH 6.2) at 37°C for 12 hours. The gel was washed briefly with water and stained with KI solution following the technique described for phosphorylase staining. The gel was allowed to stain until very dark (approximately 30 minutes) and then was transferred to tap water. In tap water, the gel underwent destaining over a period of two days. The destaining was such that the red-purple color of the amylopectin destained at a faster rate than did the blue color of the debranched zones. This technique resulted in a gel having blue zones against a clear to slightly pink background. The blue zones were taken as zones of R-enzyme activity.

2. Description and Occurrence

Isoenzymes of amylopectin-1,6-glucosidase (R-enzyme) were studied in immature endosperm (16 days following pollination) of maize lines that were homozygous for the endosperm mutant genes su_1, su_2, o_2, du_1, ae, wx, and the normal starchy genotype. The endosperm mutant genes with the exception of su_1 were incorporated into a common background stock (K55). Whole

mature dry seed extracts involving mutants ae, wx, the triply recessive mutant ae wx su_1, and su_1; the starchy inbreds Oh7 and Oh43, were also studied.

Figure 21 illustrates the R-enzyme isoenzymes found in maize. Isoenzymes A, B, and C were found in immature endosperm and whole seed extracts while isoenzymes D, E, and F were restricted to whole seed extracts. Isoenzyme A occurred independently of isoenzymes B and C and was found in the su_2 mutant endosperm. The remaining mutants all contained isoenzymes B and C. Isoenzymes D, E, and F were found in all whole seed extracts examined being strongest in the two starchy types (Oh7 and Oh43).

The carbohydrate constituents of maize endosperm mutants were examined by Creech et al., (1965). They found that the mutant wx contained nearly 100% amylopectin as a starch reserve whereas the mutant ae contained close to 50% amylose as starch reserve. The mutant du_1 contained up to 38% amylose, the normal starchy lines contained about 27% amylose, su_1 contained approximately 29% amylose and su_2 contained approximately 40% amylose. Black et al. (1966) examined the phytoglycogen contents and branching enzyme activities of several of these mutants and found that su_1 was the only line that accumulated this branched component. Double recessive du_1wx and the triply recessive ae su_1 wx also stored some phytoglycogen. One might

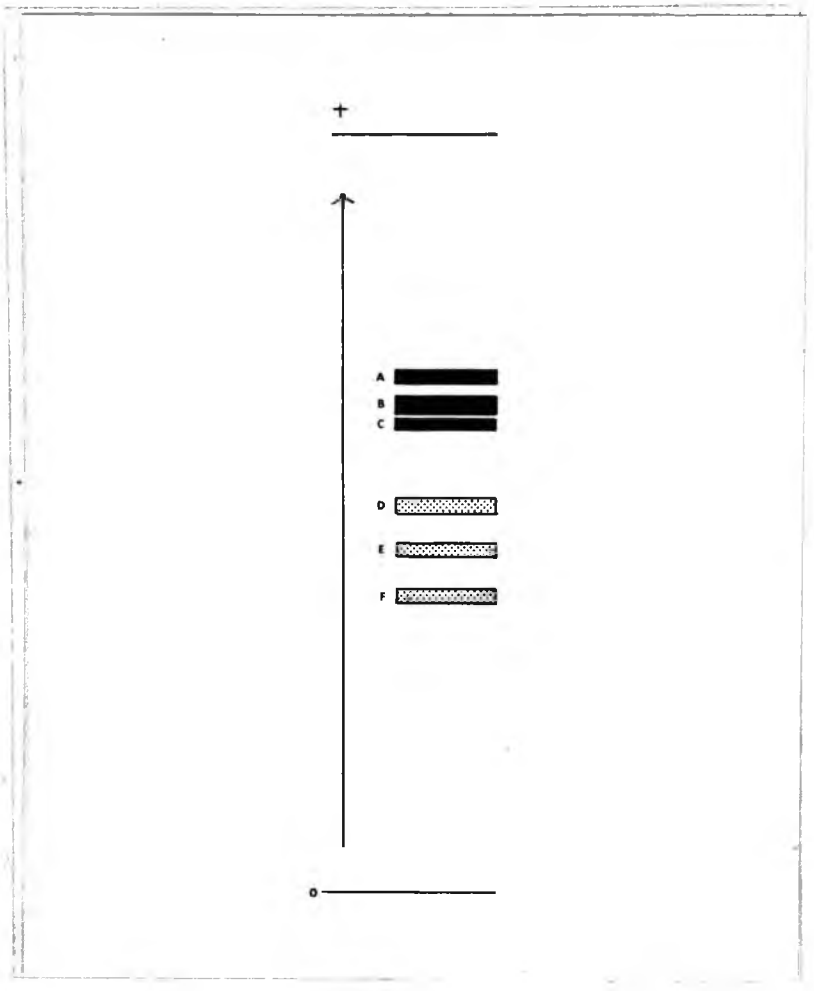

Fig. 21. An Illustration of the Amylopectin-1,6-Glucosidase Isoenzymes of Maize. Arrow indicates direction of migration towards the anode.

expect that mutants high in branched starches would contain low R-enzyme levels and that mutants low in branched starches might contain higher levels of R-enzyme. This was not found to be the case in the present study. Mutant wx, which stores 100% amylopectin, contained the same R-enzyme isoenzymes as mutant ae, which contains a large portion of the linear amylose starch. The strongest R-enzyme activity was found in the starchy inbreds Oh7 and Oh43. These normal types generally contain only about 29% amylose. No correlation can be made at present with respect to R-enzyme activity and the different proportions of branched and linear starches in the mutant endosperm lines.

AMYLASE ISOENZYMES

1. Introduction and Staining Technique

Two major types of amylases exist. The α-amylases are found in animals and in certain plant tissues at certain stages of growth. The β-amylases are most common in plants and are found in most tissues of plants. Both types of amylases attack 1,4-glucosidic bonds with the eventual release of maltose. β-amylase attacks maltodextrins at the nonreducing end, cleaving off maltose units as it works its way along the maltodextrin chain. Its activity is halted when it encounters bonds other than the 1,4-glucosidic bond (eg., 1,6-glucosidic bonds). α-amylase attacks a maltodextrin at random causing fractionation of relatively large molecules and producing a mixture of small maltodextrins. The smaller maltodextrins are also subject to attack by the α-amylase resulting in the eventual production of maltose. Like β-amylase, α-amylase cannot cleave bonds other than the 1,4-glucosidic bond. Branched maltodextrins subjected to β-amylase attack undergo limited degradation. The products of such an attack are maltose and β-limit dextrin. Likewise, a branched maltodextrin subjected to α-amylase produces α-limit dextrin and maltose.

The present study involved all of the amylases in the extracts. No attempt was made to differentiate between the two types of amylases.

Two general techniques were used in the present study to stain for amylase activity. Acrylamide gels were used due to the affinity the amylases have for starch gels. The first technique involved incorporating 0.1% starch (amylose or amylopectin) into the gel prior to electrophoresis. Following electrophoresis, the gels were incubated in 0.1M tris-citrate buffer (pH 6.2) at 37°C for one hour. The gels were then rinsed with water and stained with KI solution as described for phosphorylase staining. The amylase isoenzymes appeared as clear zones against a red-purple background in the case of incorporated amylopectin, or blue background in the case of incorporated amylose. The incorporated starches caused a small amount of change in the migration rate of the isoenzymes due to the affinities of the amylases for the incorporated starch. Good separations of the amylases were achieved, however, using only 0.1% concentrations of incorporated starch.

The second method of staining did not involve incorporation of starch in the gel. The gels were incubated in a 2% starch-buffer solution for 2 hours at 37°C. Two grams amylose were dissolved by heating in 0.1M tris-citrate buffer (pH 6.2). The mixture was cooled and the gel was immersed and incubated. The gel was stained using the KI solution technique described for

phosphorylase staining. Here, like the previous stain, the amylase isoenzymes appeared as clear zones against a blue background.

Phosphorylase isoenzymes were not stained using this technique due to the absence of phosphate.

2. Description and Occurrence

Amylase isoenzymes following the staining techniques described above were examined in immature endosperm (16 days following pollination) of homozygous su_1 lines and in endosperm, scutellum, root, first leaf, and coleoptile tissues of 7-day old seedlings of the same inbred. Figure 22 illustrates the amylase isoenzymes found in the maize tissues. Eight well defined isoenzymes were found along with several large zones extending from the origin towards the anode that were smeared and difficult to classify as isoenzymes. Immature endosperm was found to contain four distinct isoenzymes and a small amount of clearing at the origin. Isoenzyme A of the immature endosperm (Figure 22) was also found in the endosperm and scutellum tissues of the seven-day old seedlings. Isoenzyme G of the immature endosperm corresponded to the single amylase isoenzyme found in the root, first leaf, and coleoptile tissues of the seven-day old seedlings. The mature endosperm of the seven-day old seedling had an amylase isoenzyme not found in other tissues (designated B in

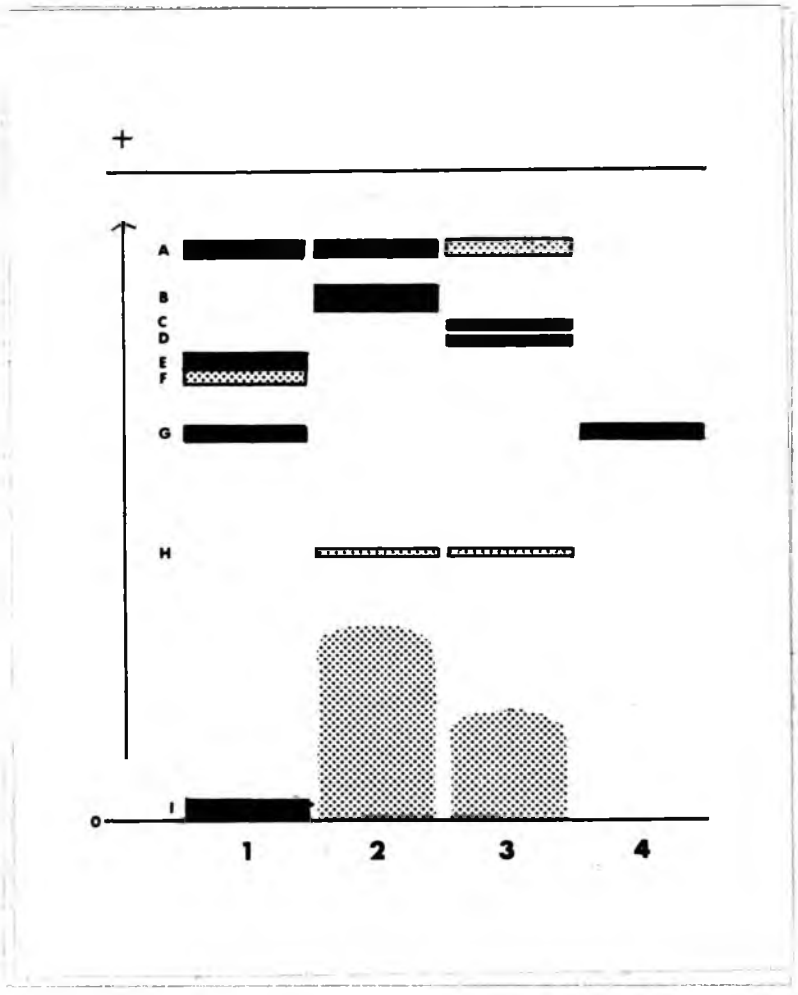

Fig. 22. An Illustration of the Amylase Isoenzymes of Maize. Arrow indicates direction of migration towards the anode.

*(1) immature endosperm, (2) endosperm, (3) scutellum, (4) root, plumule, and coleoptile from 7-day old seedlings of inbred AA3 (Haw.).

Figure 22). The scutellum of the seven-day old seedlings contained two isoenzymes (C and D) not found in other tissues. Classification of the amylases as to alpha and beta types was not attempted.

3. Changes During Germination and Development

Inbred AA3 was used in a developmental study of the amylase isoenzymes. Seed of inbred AA3 was soaked for 24 hours and then transferred to petri dishes containing moistened filter paper. The 24-hour soaking period was considered the first day of germination. On the third day of germination, the petri dishes were removed from the dark where they had been stored, following sowing of the seed, and placed on the benchtop in the laboratory. Endosperm and scutellum tissues were separated and washed with distilled water. Tissue samples from 1, 2, 3, 4, 5, 6, and 7-day old seedlings were extracted and subjected to electrophoresis. Amylase activity following the technique described above was analyzed.

Figure 23 illustrates the isoenzymic patterns noted in this study. As can be seen from the figure, all of the isoenzymes found in these tissues were present throughout the germination period. The only change in pattern noted was an increase in isoenzyme intensity as the seedling aged or as germination proceeded. Isoenzyme A in the scutellum appeared to decrease slightly in intensity over this period.

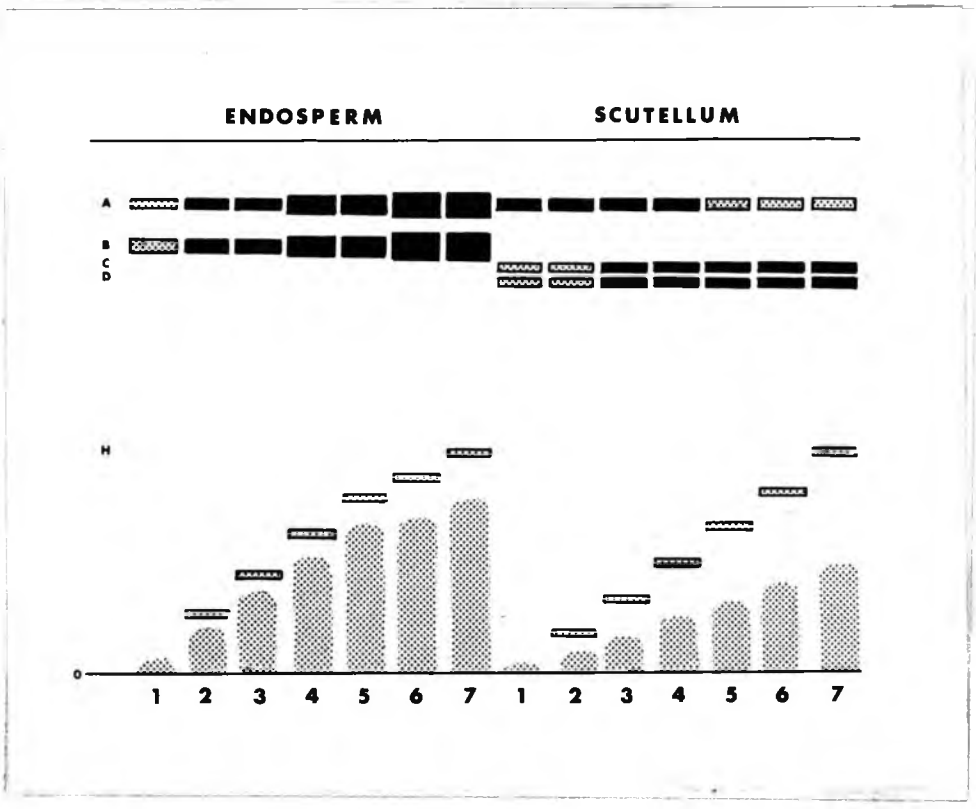

Fig. 23. Changes in the Amylase Isoenzyme Patterns of Endosperm and Scutellum Tissues from Germinating Seed of Inbred AA3 (Haw.) During Seedling Development.

*Extracts from (1)-(7) day old seedlings.

SUMMARY

The primary objective of the study was that of the genetic analysis of polymorphisms observed, and ten different controlling loci have been described. Gel electrophoretic studies were conducted for esterase, L-aspartate:2-oxoglutarate aminotransferase (GOT), and several carbohydrase enzymes in Zea mays. Additional studies included tissue specificity of the isoenzymes, the development of the isoenzymic spectrum in developing plant tissues, substrate specificities of the isoenzymes, and inhibition and activation of the isoenzymes by a variety of chemical compounds.

Esterase: Approximately 30 anodal esterase isoenzymes were found in the 17 tissues of maize analyzed in the present study. Three isoenzymes were found to be restricted to immature endosperm, two were restricted to pollen, and a single group of isoenzymes was restricted to seedling root tissue. Five isoenzymes were found in all tissues examined. The other isoenzymes were found in several tissues.

Isoenzymic patterns changed with development and growth. Several isoenzymes were found to appear or disappear during germination and seedling development.

Several substrates were tested with extracts from select inbred lines in an attempt to determine the substrate specificity of the isoenzymes. Generally speaking, only the shorter chain

substrates were hydrolyzed at a significant rate. Several isoenzymes were able to hydrolyze the lipid-like longer chained substrates. Classification on the basis of lipase and esterase was not applied due to overlapping substrate specificities.

Approximately 40 organic and inorganic compounds were tested as inhibitors and activators. Specific inhibition was achieved with fluoride, permanganate, EDTA, pCMB, and a number of organophosphate and carbamate compounds. Specific activation was achieved with atropine. Classification of the esterases on the basis of inhibition was unsuccessful due to differential sensitivities by the isoenzymes towards several organophosphates.

Genetic analysis of the esterases led to the description of nine new controlling loci. The gene symbol Oe_4 was postulated as designating the regulatory portion of a 'prime allele' controlling the E_4 esterase locus. Interaction between the Oe_4 portion of the E_4 locus and functional allele of a second regulatory locus (Re_4) resulted in cessation of enzyme production in lines containing the 'prime allele' but not in lines containing a 'standard allele'.

The E_5 esterases were controlled by two independently segregating loci (E_5-I and E_5-II). The mechanism of control appeared to be one of recessive complimentation.

The E_6 through E_{10} esterases are monogenic in behavior and contained two or three alleles at each locus.

L-aspartate:2-oxoglutarate aminotransferase: Thirteen GOT isoenzymes were found in the tissues studied. All migrated towards the anode at pH 8.2. Tissue specificity was noted for several of the isoenzymes and four isoenzymes were found in all tissues. Changes in the isoenzyme spectrum of developing tissues were noted.

All 13 isoenzymes were specific for the L-form of aspartic acid. Three isoenzymes were able to utilize keto acids other than ketoglutarate while the remaining isoenzymes were specific for ketoglutarate.

Genetic analysis showed that two isoenzymes were controlled by the same locus (Ta_1). These two isoenzymes formed a hybrid isoenzyme in heterozygotes.

Carbohydrases: Four phosphorylase isoenzymes were found in maize and four were found in potato tubers. Several of the maize isoenzymes appeared to synthesize amylose-like polysaccharide in the absence of supplied primer materials. A single isoenzyme from potato had the same capacity. The 'primer-free' phosphorylases from maize and potato were respondent to primers as indicated by increased levels of synthesis in the presence of primers.

Amylopectin-1,6-glucosidase (R-enzyme) activity was found in several tissues of maize. Six well defined isoenzymes were found.

Several amylase isoenzymes were found in different tissues of maize. Several of the isoenzymes exhibited tissue specificity. Endosperm and scutellum tissue showed increased amylase activity with increased age of germinating seed.

LITERATURE CITED

Abdullah, M., Fischer, E. H., Qureshi, M. Y., Slessor, K. N., and Whelan, W. J. 1965. Requirement of rabbit-muscle glycogen phosphorylase for primer. Biochem. J. 97:9 p.

Albaum, H. G. and Cohen, P. P. 1943. Transamination and protein synthesis in germinating oat seedlings. J. Biol. Chem. 149:19-27.

Aldridge, W. N. 1953a. Serum esterases 1. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate, and butyrate, and a method for their determination. Biochem. J. 53:110-117.

_____. 1953b. Serum esterases 2. An enzyme hydrolysing diethyl p-nitrophenyl phosphate (E600) and its identity with the A-esterase of mammalian sera. Biochem. J. 53: 117-124.

Andronescu, D. I. 1919. Germination and further development of the embryo of Zea mays separated from the endosperm. Amer. J. Bot. 6:443-452.

Beevers, H. 1961. Respiratory Metabolism in Plants. Row Patterson, Evanston, Ill., 1961.

_____. 1961. Metabolic production of sucrose from fat. Nature 191:433-436.

Beevers, L., Schroder, L. E., Flesher, D., and Hageman, R. H. 1965. The role of light in the induction of nitrate reductase in radish cotyledons and maize seedlings. Plant Physiol. 40:691-698.

Bergmann, F., Segal, R., and Rimon, S. 1957. A new type of esterase in hog-kidney extract. Biochem. J. 67:481-486.

Black, R. C., Loerch, J. D., McArdle, F. J., and Creech, R. G. 1966. Genetic interactions affecting maize phytoglycogen and phytoglycogen-forming branching enzyme. Genet. 53:661-668.

Bolkova, A., Novak, S., and Skorepa, J. 1960. Differences between liver aliesterase and serum post-heparin esterase. Sbornik lekarsky 62:278-290.

Bone, D. H. and Fowden, L. 1960. Transamination and oxidation of some amino-acids by mung-bean mitochodria. J. Exper. Bot. 11:104-115.

Bonner, J. and Varner, J. E. 1965. Plant Biochemistry, Academic Press, New York.

Brewbaker, J. L., Upadhya, M. D., Makinen, Y., and Macdonald, T. 1968. Isoenzyme polymorphism in flowering plants III. Gel electrophoretic methods and applications. Physiol. Plant. 21:930-940.

Brown, D. H., Illingworth, B., and Cori, C. F. 1961. The mechanism of de novo synthesis of polysaccharide by phosphorylase. Proc. Nat. Acad. Sci. (U. S.) 47:479-485.

Brown, H. T. and Morris, G. H. 1890. Researches on the germination of some Graminae. J. Chem. Soc., Trans. 57:458-528.

Brown, R. 1946. Studies on germination and seedling growth III. Early growth in relation to certain aspects of nitrogen metabolism in the seedling of barley. Ann. Bot. 10:73-96.

Bulen, W. A. 1956. The isolation and characterization of glutamic dehydrogenase from corn leaves. Arch. Biochem. Biophys. 62:173-183.

Cimasoni, G. 1966. Inhibition of cholinesterase by fluoride in vitro. Biochem. J. 99:133-137.

Cori, C. F., Colowick, S. P., and Cori, G. T. 1937. The isolation and synthesis of glucose-1-phosphoric acid. J. Biol. Chem. 121:465-477.

Cori, G. T. and Cori, C. F. 1943a. Crystalline muscle phosphorylase IV. Formation of glycogen. J. Biol. Chem. 151:57-63.

Cori, C. F., Cori, G. T., and Green, A. A. 1943b. Crystalline muscle phosphorylase III. Kinetics. J. Biol. Chem. 151:39-55.

Cori, G. T. and Green, A. A. 1943c. Crystalline muscle phosphorylase II. Prosthetic group. J. Biol. Chem. 151: 31-38.

Creech, R. G. 1965. Genetic control of carbohydrate synthesis in maize endosperm. Genet. 52:1175-1186.

Cline, A. L. and Bock, R. M. 1966. Translational control of gene expression. Cold Springs Harbor Sympos. Quant. Biol. 31:321-333.

Danielson, C. E. 1951. The breakdown of high-molecular reserve proteins of peas during germination. Acta Chem. Scand. 5:551-554.

Decker, L. E. and Rau, E. M. 1963. Multiple forms of glutamic-oxaloacetic transaminase in tissues. Proc. Soc. Exper. Biol. Med. 112:144-149.

Dure, L. S. 1960a. Gross nutritional contributions of maize endosperm and scutellum to germination and growth of maize axis. Plant Physiol. 35:919-925.

_____. 1960b. Site of origin and extent of activity of amylases in maize germination. Plant Physiol. 35:929-934.

Ecobichon, D. J. 1966. Properties and classification of the soluble esterases of human brain. Canad. J. Physiol. Pharmacol. 44:225-232.

Edelman, J., Shibko, S. I., and Keys, A. J. 1959. The role of the scutellum of cereal seedlings in the synthesis and transport of sucrose. J. Exper. Bot. 10:178-189.

Ellis, R. J. and Davies, D. D. 1961. Glutamic-oxaloacetic transaminase of cauliflower. I. Purification and specificity. Biochem. J. 78:615-623.

Eranko, O., Kokko, A., and Soderholm, U. 1962. Separation of substrate-specific brain esterases by starch-gel electrophoresis. Nature 193:778-779.

Erdos, E. G. and Laswick, J. A. 1961. Arylesterase in blood: Irreversible inactivation of the plasma enzyme-II. Biochem. Pharmacol. 8:375-381.

Erdos, E. G., Debay, C. R., and Westerman, M. P. 1960. Arylesterases in blood: Effect of calcium and inhibitors. Biochem. Pharmacol. 5:173-186.

Folkes, B. F. and Yemm, E. W. 1958. The respiration of barley plants X. Respiration and the metabolism of amino acids and proteins in germinating grain. New Phytologist 57:106-131.

Fowden, L. 1967. Aspects of amino acid metabolism in plants. Ann. Rev. Plant Physiol. 18:85-106.

Fulton, M. P. and Mogey, G. A. 1954. Some selective inhibitors of true cholinesterase. Brit. J. Pharmacol. 9: 138-144.

Green, A. A. and Cori, G. T. 1943. Crystalline muscle phosphorylase I. Preparation, properties, and molecular weight. J. Biol. Chem. 151:21-29.

Green, D. E., Leloir, L. F., and Nocito, V. 1945. Transaminases. J. Biol. Chem. 161:559-582.

Hageman, R. H., Leng, E. R., and Dudley, J. W. 1967. A biochemical approach to corn breeding. Adv. Agron. 19: 45-86.

Hall, C. B. 1966. Inhibition of tomato pectinesterase by tannic acid. Nature 212:717-718.

Harley, J. L. and Beevers, H. 1963. Acetate utilization by maize roots. Plant Physiol. 38:117-123.

Harris, J. W. 1966. The E_4 esterases. Maize Genetics Cooperation News Letter 40:53-56.

_____. 1968. Location of the E_4 esterase locus on chromosome 3. Maize Genetics Cooperation News Letter 42:72-74.

Hawkins, R. D. and Gunter, J. M. 1946. Studies on cholinesterase 5. The selective inhibition of pseudo-cholinesterase in vivo. Biochem. J. 40:192-196.

Hawkins, R. D. and Mendel, B. 1949. Studies on cholinesterase 6. The selective inhibition of true cholinesterase in vivo. Biochem. J. 44:260-264.

Heilbronn, E. 1965. Action of fluoride on cholinesterase I. On the mechanism of inhibition. Acta Chem. Scand. 19: 1333-1346.

Hobson, P. N., Whelan, W. J., and Peat, S. 1950. A debranching enzyme in bean and potato. Biochem. J. 47: xxxix.

_____, _____, and _____. 1951. The enzymic synthesis and degradation of starch. Part XIV. R-enzyme. J. Chem. Soc. 1951:1451-1459.

Hofstee, B. H. J. 1960. In The Enzymes., Eds. Boyer, Lardy, and Myrback. Vol. 4, p. 485, Academic Press, New York.

Hunter, R. L. and Burstone, M. S. 1960. The zymogram as a tool for the characterization of enzyme substrate specificity. J. Histochem. Cytochem. 6:58-62.

Hunter, R. L. and Strachan, D. S. 1961. The esterases of mouse blood. Ann. N. Y. Acad. Sci. 94:861-867.

Ingle, J., Beevers, L., and Hageman, R. H. 1964. Metabolic changes associated with the germination of corn I. Changes in weight and metabolites and their redistribution in the embryo axis, scutellum, and endosperm. Plant Physiol. 39: 735-740.

Illingsworth, B., Brown, D. H., and Cori, C. F. 1961. The de novo synthesis of polysaccharide by phosphorylase. Proc. Nat. Acad. Sci. (U. S.) 47:469-478.

Jacob, F. and Monod, J. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3:318-356.

James, W. O. and James, A. L. 1940. The respiration of barley germinating in the dark. New Phytologist 37:455-473.

Kamogawa, A., Fukui, T, and Nikuni, Z. 1968. Potato α-glucan phosphorylase: Crystallization, amino acid composition and enzymatic reaction in the absence of added primer. J. Biochem. (Japan) 63:361-369.

Keay, L. and Crook, E. M. 1965. Effect of metal ions on hog liver esterase. Arch. Biochem. Biophys. 111:626-634.

Kessler, E. 1964. Nitrate assimulation by plants. Ann. Rev. Plant Physiol. 15:57-72.

Kitto, G. B., Kottke, M. E., Bertland, L. H., Murphey, W. H., and Kaplan, N. O. 1967. Studies on malate dehydrogenase and aspartate aminotransferase from Neurospora crassa. Arch. Biochem. Biophys. 121:224-232.

Kitto, G. B., Wassarman, P. M., and Kaplan, N. O. 1966. Enzymatically active conformers of mitochondrial malate dehydrogenase. Proc. Nat. Acad. Sci. (U. S.) 56:578-585.

Koelle, G. B. 1950. The histochemical differentiation of types of cholinesterase and their localization in tissues of the cat. J. Pharmacol. Exper. Ther. 100:158-179.

Komma, D. J. 1963. Characteristics of the esterases of human cells grown in vitro. J. Histochem. Cytochem. 11:618-623.

Kretovich, W. L. 1965. Some problems of amino acid and amide biosynthesis in plants. Ann. Rev. Plant Physiol. 16:141-154.

Latner, A. L. 1967. Isoenzymes. Adv. Clin. Chem. 9:69-163.

Leonard, M. J. K. and Burris, R. H. 1947. A survey of transaminases in plants. J. Biol. Chem. 170:701-709.

Lewis, A. A. M. and Hunter, R. L. 1966. The effect of fat ingestion on the esterase isozymes of intestine, intestinal lymph, and serum. J. Histochem. Cytochem. 14:33-39.

Macdonald, T. 1967. Pollen esterase isozymes. Maize Genetics Cooperation News Letter 41:60-62.

MacWilliam, I. C. 1958. Heterogeneity of R-enzyme. Nature 181:1143-1144.

MacWilliam, I. C. and Harris, G. 1959. The separation of limit dextrinase from R-enzyme and aspects of the activities of the separated enzymes. Arch. Biochem. Biophys. 84: 442-454.

Malhotra, R. C. 1934. Chemistry of corn seed germination. Cereal Chem. 11:105-109.

Markert, C. L. and Møller, F. 1959. Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. Proc. Nat. Acad. Sci. (U. S.) 45:753-763.

Martinez-Carrion, M., Riva, F., Turano, C., and Fasella, P. 1965. Multiple forms of supernatant glutamate-aspartate transaminase from pig heart. Biochem. Biophys. Res. Comm. 20:206-211.

Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F., and Fasella, P. 1967. Isolation and characterization of multiple forms of glutamate-aspartate aminotransferase from pig heart. J. Biol. Chem. 242: 2397-2409.

Marton, A. V. and Kalow, W. 1960. Inhibitors of aromatic esterase of human serum. Biochem. Pharmacol. 3:149-154.

Meister, A. 1957. Biochemistry of the Amino Acids. Academic Press, New York.

Meyers, D. K., Kemp, A., Tol, J. W., and DeJonge, M. H. T. 1957. Studies on aliesterase. 6. Selective inhibitors of the esterases of brain and saprophytic mycobacteria. Biochem. J. 65:232-241.

Mounter, L. A. and Mounter, M. E. 1962. Specificity and properties of wheatgerm esterase. Biochem. J. 85:576-580.

Mounter, L. A. and Whittaker, V. P. 1953. The effect of thiol and other group-specific reagents on erythrocyte and plasma cholinesterases. Biochem. J. 53:167-173.

Moustafa, L. A., Hassan, A., and Zayed, S. M. A. D. 1966. Translocation and degradation of 14-C-labelled Seven in cotton plant. Z. Naturforschg. 21b:1060-1062.

Nachlas, M. M. and Seligman, A. M. 1949. Evidence for the specificity of esterase and lipase by the use of three chromatographic substrates. J. Biol. Chem. 181:343-355.

Nason, A. 1950. The distribution and biosynthesis of niacin in germinating corn. Amer. J. Bot. 37:612-622.

Oaks, A. 1965. The regulation of nitrogen loss from maize endosperm. Canad. J. Bot. 43:1077-1082.

Oaks, A. and Beevers, H. 1964a. The requirement for organic nitrogen in Zea mays embryos. Plant. Physiol. 39: 37-43.

Oaks, A. and Beevers, H. The glyoxylate cycle in maize scutellum. Plant Physiol. 39:431-434.

O'Brien, R. D. 1966. Mode of action of insecticides. Ann. Rev. Entomol. 11:369-402.

Pastor, E. F. and Fennell, R. A. 1959. Some observations on the esterases of Tetrahymena pyriformis W. II. Some factors affecting aliesterase and cholinesterase activity. J. Morphol. 104:143-157.

Peat, S., Whelan, W. J., Hobson, P. N., and Thomas, G. J. 1954. The enzymic synthesis and degradation of starch. Part XIX. The action of R-enzyme on glycogen. J. Chem. Soc. 1954:4440-4445.

Ries, S. K., Chimel, H., Dilley, D. R., and Filner, P. 1967. The increase in nitrate reductase activity and protein content of plants treated with simazine. Proc. Nat. Acad. Sci. (U. S.) 58:526-532.

Ritenour, G. L., Joy, K. W., Bunning, J., and Hageman, R. H. 1967. Intracellular localization of nitrate reductase, nitrite reductase, and glutamic acid dehydrogenase in green leaf tissue. Plant Physiol. 42:233-237.

Scandalios, J. G. 1964. Tissue-specific isozyme variation in maize. J. Hered. 55:281-285.

Schwark, W. S. and Ecobichon, D. J. 1967. Characterization of rat liver and kidney esterases. Canad. J. Biochem. 45: 451-455.

Schwartz, D. 1960. Genetic studies on mutant enzymes in maize: Synthesis of hybrid enzymes by heterozygotes. Proc. Nat. Acad. Sci. (U. S.) 46:1210-1215.

Schwartz, D. 1962a. Genetic studies on mutant enzymes in maize II. On the mode of synthesis of the hybrid enzymes. Proc. Nat. Acad. Sci. (U. S.) 48:750-756.

_____. 1962b. Genetic studies on mutant enzymes in maize III. Control of gene action in the synthesis of pH 7.5 esterases. Genet. 47:1609-1615.

_____. 1964a. Regulation of gene action in maize. Genetics Today, Proc. XI Internat. Congr. Genet., Sept. 1963.

_____. 1964b. A second hybrid enzyme in maize. Proc. Nat. Acad. Sci. (U.S.) 51:602-605.

_____. 1964c. Genetic studies on mutant enzymes in maize IV. Comparison of the pH 7.5 esterases synthesized in seedling and endosperm. Genet. 49:373-377.

_____. 1964d. Genetic studies on mutant enzymes in maize V. In vitro interconversion of allelic isozymes. Proc. Nat. Acad. Sci. (U. S.) 52:222-226.

_____. 1964e. Tissue specificity and control of gene action. Erwin-Baur-Gedachtnis varlesungen III, 1963, Akademie-Verlag. Berlin, 1964.

_____, Fuchsman, L., and McGrath, K. H. 1965. Allelic isozymes of the pH 7.5 esterases in maize. Genet. 52:1265-1268.

_____. 1965. Genetic studies on mutant enzymes in maize VI. Elimination of allelic isozyme variation by glyceraldehyde treatment. Genet. 52:1295-1302.

_____. 1967. E_1 esterase isozymes in maize: On the nature of the gene-controlled variation. Proc. Nat. Acad. Sci. (U. S.) 58:568-575.

Shannon, L. M. 1968. Plant isoenzymes. Ann. Rev. Plant Physiol. 19:187-210.

Smithies, O. 1955. Zone electrophoresis in starch gel: Group variations in the serum proteins of normal human adults. Biochem. J. 61:629-641.

Steward, F. C. and Pollard, J. K. 1957. Nitrogen metabolism in plants: Ten years in retrospect. Ann. Rev. Plant Physiol. 8:65-114.

Stumpf, P. K. 1951. Transaminase in higher plants. Fed. Proc. 10:256.

Stumpf, P. K. and Barber, G. A. 1956. Fat metabolism in higher plants VII. β-oxidation of fatty acids by peanut mitochondria. Plant Physiol. 31:304-308.

Thorne, C. B., Gomez, C. G., and Housewright, R. D. 1955. Transamination of D-amino acids by Bacillus subtilis. J. Bacteriol. 69:357-362.

Thorne, C. B. and Molnar, D. M. 1955. D-amino acid transamination in Bacillus anthracis. J. Bacteriol. 70:420-426.

Toole, E. H. 1924. The transformations and course of development of germinating maize. Amer. J. Bot. 11:325-350.

Tsai, C. Y. and Nelson, O. E. 1968. Phosphorylase I and II of maize endosperm. Plant Physiol. 43:103-112.

Tweedy, J. A. and Ries, S. K. 1967. Effect of simazine on nitrate reductase activity in corn. Plant Physiol. 42:280-282.

van Asperen, K. 1960. Toxic action of organophosphorous compounds and esterase inhibition in houseflies. Biochem. Pharmacol. 3:136-146.

Virtanen, A. I. 1961. Some aspects of amino acid synthesis in plants and related subjects. Ann. Rev. Plant Physiol. 12: 1-12.

Vogel, H. J. and Vogel, R. H. 1967. Regulation of protein synthesis. Ann. Rev. Biochem. 36:519-538.

Watkins, J., Normansell, D. E., and Gilbert, G. A. 1965. Presence of maltodextrins in potato phosphorylase preparations. Nature 207:857-858.

Webb, E. C. 1964. The nomenclature of multiple enzyme forms. Experimentia 20:592.

Wiessman, G. S. 1959. Influence of ammonia and nitrate on the proteins and free amino acids in shoots of wheat seedlings. Amer. J. Bot. 46:339-346.

West, E. S., Todd, W. R., Mason, H. S., and Van Bruggen, J. T. 1966. Textbook of Biochemistry. MacMillan Company, New York.

Wilkinson, J. H. 1966. Isoenzymes. J. B. Lippincott Company, Philadelphia, Pa.

Wilson, D. G., King, K. W., and Burris, R. H. 1954. Transaminase reactions in plants. J. Biol. Chem. 208: 863-874.

Yemm, E. W. and Folkes, B. F. 1958. The metabolism of amino acids and proteins in plants. Ann. Rev. Plant Physiol. 9:245-280.

Zeller, A. 1957. Die Mobilisierung der Fette bei der Keimung. In: Encyclopedia of Plant Physiology. Springer-Verlag, Berlin. VII:323-353.